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Bidirectional Interactions Between Indomethacin and the Murine intestinal Microbiota

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Abstract
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The vertebrate intestinal microbiota have a highly diverse enzymatic system and hence have been implicated in the metabolism of various xenobiotics, including clinically important drugs. The composition of intestinal microbiota exhibits considerable inter-individual differences, and dysbiosis has been associated with the pathogenesis of many diseases.

The present study is designed to elucidate the interactions between the microbiota and indomethacin, an NSAID that inhibits cyclooxygenases (COX) -1 and 2. At a dose that caused small intestinal damage, orally administered indomethacin was distributed in the circulation and in the intestinal tract, and suppressed the production of both COX-1 and COX-2 derived prostaglandins in vivo. Deep sequencing analysis showed that indomethacin altered the composition and diversity of the large intestinal and fecal microbiota, shifting it towards a pro-inflammatory phenotype. Depletion of the intestinal microbiota by antibiotic treatment increased indomethacin elimination, and decreased its half-life and volume of distribution. This involves the de-glucuronidation mediated by the β-glucuronidase-expressing bacteria in the intestinal microbiota. Therefore, the intestinal microbiota may be a potential source of inter-individual variability in the pharmacokinetics and/or pharmacodynamics of indomethacin.

This study also found that the total fecal microbial load, as well as the relative abundances of Bacteroidetes and Firmicutes oscillated during the light-dark cycle. This oscillation requires a functional host circadian clock, since deletion of Bmal1, a gene encoding a core molecular clock component, abolished this rhythmicity. The fecal microbiota composition was also altered by Bmal1-deletion. In addition, the relative abundances of several β-glucuronidase-expressing bacteria oscillate diurnally, suggesting the involvement of intestinal microbiota in the chronopharmacology of indomethacin.

The indomethacin-microbiota interactions described here provide candidate mechanisms for pathogenesis of GI toxicity, individualized drug responses, as well as the circadian variation of drug kinetics and effects.

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Xue Liang

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DEDICATION

This thesis is dedicated to my parents,

Zhenxiang Liang & Yanping Zhao

For their endless love, support, and encouragement.

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ABSTRACT

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Xue Liang

Garret A. FitzGerald, M.D.

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The vertebrate intestinal microbiota have a highly diverse enzymatic system and hence have been implicated in the metabolism of various xenobiotics, including clinically important drugs. The composition of intestinal microbiota exhibits considerable inter-individual differences, and dysbiosis has been associated with the pathogenesis of many diseases.

The present study is designed to elucidate the interactions between the microbiota and indomethacin, an NSAID that inhibits cyclooxygenases (COX) -1 and 2. At a dose that caused small intestinal damage, orally administered indomethacin was distributed in the circulation and in the intestinal tract, and suppressed the production of both COX-1 and COX-2 derived prostaglandins *in vivo*. Deep sequencing analysis showed that indomethacin altered the composition and diversity of the large intestinal and fecal

microbiota, shifting it towards a pro-inflammatory phenotype. Depletion of the intestinal microbiota by antibiotic treatment increased indomethacin elimination, and decreased its half-life and volume of distribution. This involves the de-glucuronidation mediated by the β-glucuronidase-expressing bacteria in the intestinal microbiota. Therefore, the intestinal microbiota may be a potential source of inter-individual variability in the pharmacokinetics and/or pharmacodynamics of indomethacin.

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The indomethacin-microbiota interactions described here provide candidate mechanisms for pathogenesis of GI toxicity, individualized drug responses, as well as the circadian variation of drug kinetics and effects.

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Chapter 1 General Introduction

1.1 Human gut microbiome

The human body is home to a large collection of microbes, including bacteria, archaea, viruses, and unicellular eukaryotes, collectively referred to as the microbiome. Among them, bacteria are more intensely studied and hence better understood. It has been estimated that the human intestinal tract harbors over 100 trillion (10^{14}) bacteria grouped in about 1000 species (Bengmark 1998, Ley, Peterson & Gordon 2006, Zhu et al. 2011), accounting for up to 98% of the intestinal microbiome (Manichanh et al. 2012). This collection of microbes is estimated to have a combined genome size exceeding that of humans by at least two orders of magnitude (Ley, Peterson & Gordon 2006).

Although virtually all surfaces of the human body are colonized by bacteria, the most heavily colonized organ is the gastrointestinal tract, and the density in the colon reaches 10^{11} to 10^{12} per milliliter (Whitman, Coleman & Wiebe 1998). The intestinal bacteria contribute greatly to host physiology, including the maintenance of mucosal structure (Stappenbeck, Hooper & Gordon 2002), host defense against pathogens (Bengmark 1998), the activation of host immune responses (Littman, Pamer 2011), fermentation of dietary fiber (Sekirov et al. 2010), vitamin production for the host (such as biotin and vitamin K) (Stevens, Hume 1998), metabolism of peptides and proteins (Farthing 2004), and metabolism of xenobiotics, including drugs (Illing 1981, Bakke, Gustafsson 1986, Rowland et al. 1986).

The intestinal microbiota are dominated by Bacteroidetes and Firmicutes, which are just two of the 70 known phyla of Bacteria. Other minor constituents, including Actinobacteria, Proteobacteria and Verrucomicrobia, account for about 10% of the intestinal microbiota (Turnbaugh et al. 2007). However, the relative proportions of these phyla are different among different individuals (Eckburg et al. 2005, Arumugam et al. 2011a). Furthermore, inter-individuality is also evident at lower taxonomic levels—the species presented in the intestine vary across individuals. Recently, attempts have been made to determine whether human beings share a core microbiota at species level. Several species are found to be present across individuals, such as *Faecalibacterium prausnitzii, Roseburia intestinalis* and *Bacteroides uniformis* (Qin et al. 2010). Yet in some individuals these phyla only account for less than 0.5% of the total microbes presented in the intestine (Turnbaugh et al. 2009). Variation even exists between genetically identical twins (Turnbaugh et al. 2010). Individuality of the gut microbiota composition was found to be shaped by multiple environmental and host genetic factors (Benson et al. 2010a). Hence, with about 6.7 billion human beings living on the Earth under various geographic, nutritional, and other conditions, the idea of a core microbiota among individuals is unlikely.

Variability in the intestinal microbiota composition across individuals is driven by a variety of factors, including host genetics (Benson et al. 2010b), ageing (Biagi et al. 2010, Agans et al. 2011), lifestyle (Annalisa et al. 2014), pet ownership (Song et al. 2013), diet (Wu et al. 2011, Zoetendal, de Vos 2014), and time of day (Thaiss et al. 2014, Zarrinpar et al. 2014). For example, the intestinal microbiota composition of children living in

Europe is different from that of children living in a rural African village in Burkina Faso (De Filippo et al. 2010). Moreover, the intestinal microbiota composition of both children and adults from the United States is very different from that of children and adults from Malawi and the Amazonas states of Venezuela (Yatsunenko et al. 2012).

The idea of enterotypes was developed, where the population was characterized by the dominance of *Bacteroides*, *Prevotella* or *Ruminococcus*, respectively, according to their relative proportions (Arumugam et al. 2011b). This concept is still under debate, since several later studies failed to reproduce the same clustering in detail (Wu et al. 2011, Yatsunenko et al. 2012). However, variability in the human intestinal microbiota composition is still evident. For instance, although not having identified all three enterotypes, a study of 98 healthy adults from the United States documented clustering of fecal communities at the levels of *Bacteroides* and *Prevotella* (Wu et al. 2011), recapitulating two of the original three enterotypes.

The composition of intestinal microbiota is also affected by the physiological and disease state of host, and disruptions in the microbiota have been associated with obesity (Ley et al. 2006a, Turnbaugh et al. 2008, Zhao 2013), malnutrition (Kau et al. 2011), Crohn's disease (Dicksved et al. 2008), inflammatory bowel disease (Frank et al. 2007), neurological disorders (Gonzalez et al. 2011), and cancer (Lampe 2008, Zhu et al. 2011, Chen et al. 2012, Zhu et al. 2014, Akin, Tozun 2014). Differences in the diversity, composition and function of the intestinal microbiota have been documented in these disease states when compared to healthy subjects. Furthermore, the use of antibiotics also affects the intestinal microbiota. For example, in healthy volunteers antibiotic treatment

led to major shifts in the dominant bacterial taxa within 24 hours of treatment, and in some subjects the changes persisted over a long time—from 2 months to 2 years (De La Cochetiere et al. 2005, Jernberg et al. 2007).

1.2 Microbial metabolism of drugs

Xenobiotics undergo the enzymatic conversion from lipophilic compounds that are readily absorbed into hydrophilic products that are excreted. Although liver is the major site, these conversions occur in other tissues, such as the gastrointestinal tract. Enzymes involved in xenobiotic metabolism include cytochrome P450 (CYP450) enzymes, carbonyl reductases, carboxylesterases, oxidase, reductase, hydrolase, as well as many transferases. Since the intestinal microbiome is highly diverse in enzymatic activities, it can metabolize many compounds. For example, more than 30 approved drugs have been identified as direct substrates for colonic bacteria to date, including digoxin, sulfinpyrazone, clonazepam, L-dopa, 5-aminosalicylic acid, azetirelin (Sousa et al. 2008). Bacterial-mediated metabolism of drugs can be substantial. Co-administration of the antiviral drug, sorivudine with the prodrug for 5-Fluorouracil (5-FU) was implicated in 18 deaths (Okuda et al. 1998). This is attributable to the inhibition of hepatic dihydropyrimidine dehydrogenase, a key enzyme regulating the systemic 5-FU level, by a bacteria-derived metabolite from sorivudine.

Three mechanisms through which the gut microbiome performs biotransformation of drugs have been suggested. First, some species directly metabolize drugs, for example, by hydrolysis, glutathione conjugation and deamination (Sousa et al. 2008). For example,

the prodrug sulfasalazine, prescribed for ulcerative colitis, is catalyzed by azoreductase encoded by colonic bacteria, releasing the active anti-inflammatory component 5-aminosalicylic acid (Peppercorn, Goldman 1972). Bacteria-mediated biotransformations can also inactivate drugs, as in the case of the cardiac glycoside digoxin. *Eubacterium lentum*, a common anaerobe of the human colonic microbiota, is capable of converting digoxin to reduced derivatives that are pharmacologically inactive (Saha et al. 1983, Haiser et al. 2013, Haiser et al. 2014). Second, the intestinal microbiota can produce biologically active metabolites that are capable of competing with drugs for host-encoded catabolic enzymes. For example, bacteria-derived p-cresol can compete with acetaminophen for cytosolic sulfotransferase (Clayton et al. 2009). Third, the gut microbiota may modulate host drug metabolizing enzymes, such as cytochrome P450s and phase II conjugating enzymes. A recent study found a significant difference in colonic levels of several phase II xenobiotic-metabolizing enzymes including glutathione transferases, epoxide hydrolases, and sulfotransferases (Meinl et al. 2009).

1.3 NSAIDs and cyclooxygenases

The nonsteroidal anti-inflammatory drugs, or NSAIDs, are widely used anti-inflammatory, analgesic, and antipyretic drugs. In the United States, annual prescription of these NSAIDs approaches 111 million, at a cost of $ 4.8 billion. These drugs also account for up to 60% of the over-the-counter analgesic market (Laine 2001).

NSAIDs act by inhibiting the cyclooxygenase (COX) enzymes, which are also called prostaglandin G/H synthases. They are bi-functional enzymes which successively

metabolize arachidonic acid (AA) to cyclic endoperoxide prostaglandins G (PGG) and H (PGH), as shown in Figure 1.1.

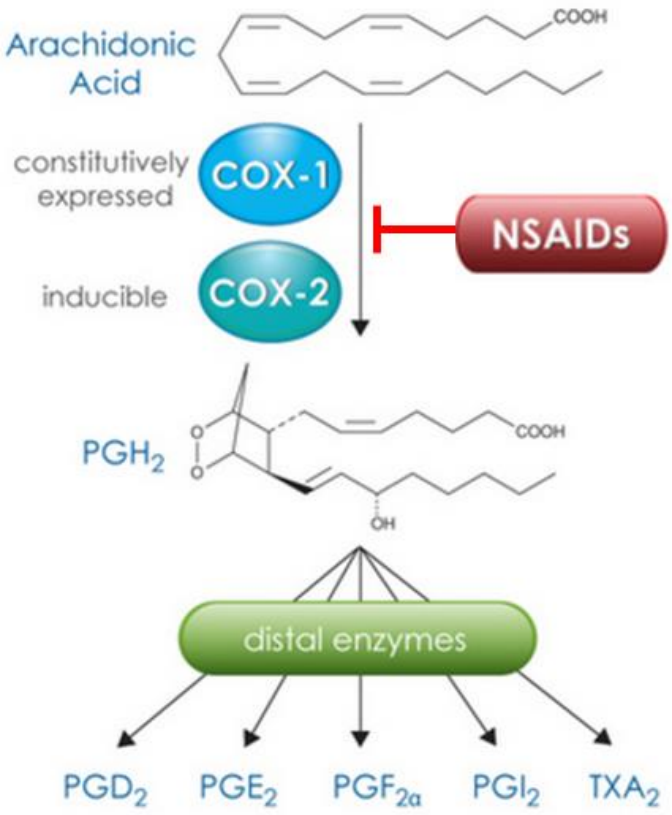

Figure 1.1 Metabolic pathways of prostanoids. Arachidonic acid (AA) is metabolized by COX-1 and COX-2 enzymes to produce PGH_2, which is metabolized by isomerases and synthases into PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2. NSAIDs act by inhibiting COX enzymes to achieve their pharmacological effects.

There are two isoforms of cyclooxygenases, COX-1 and COX-2 (Smith, Garavito & DeWitt 1996). These two isoforms share 61% amino acid identity, and their crystal

structures are remarkably similar (FitzGerald, Loll 2001). Both isoforms have cyclooxygenase activity which oxygenates and cyclizes unesterified AA to form PGG_2, and hydroperoxidase activity which converts PGG_2 to PGH_2 (Smith, Langenbach 2001). COX-1 and COX-2 have comparable Michaelis constant (K_m) values for AA, despite some evidence that COX-2 is activated at lower peroxide concentrations than COX-1 (Chen, Pawelek & Kulmacz 1999). COX-1 is constitutively expressed in most cells, whereas COX-2 expression is induced by cytokines, shear stress, and growth factors (Smith, Langenbach 2001). Therefore, COX-1 is mainly contributing PGs to housekeeping functions, and COX-2 is mainly contributing those relevant to inflammation and cancer (Murata et al. 1997, Goulet et al. 2004).

1.4 Prostanoids

Prostanoids are eicosanoids (derived from the Greek word εικωσι for 20) -- lipid mediators and products of AA, which is an unsaturated fatty acid constituent of cell membranes (C20:4). They are produced in a wide range of tissues, and play essential roles in human health and disease, such as blood clotting, wound healing, immune responses, bone metabolism, nerve growth and development, and inflammation (Ulrich, Bigler & Potter 2006). Prostaglandins are not stored in cells, but are synthesized and released when cells are activated (Funk 2001).

AA is the most abundant precursor of eicosanoids in humans; it is either derived from dietary linoleic acid or ingested directly as a dietary constituent. Upon cell stimulation, AA is released from phospholipids in cellular membranes, and metabolized rapidly to

oxygenated products by several enzyme systems, including cyclooxygenase (COX), lipoxygenase (LOX), and CYPs (Grosser, Yu & FitzGerald 2010).

As shown in Figure 1.1, COX-1 and COX-2 catalyze the biotransformation of AA to PGG_2 and PGH_2. The latter is then metabolized through isomerases and synthases effect into terminal prostanoids, including PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2. Following their synthesis, these prostanoids are released from cells through facilitated transport (Schuster 2002), such as via the prostaglandin transporter, to exert autocrine or paracrine actions on a family of membrane expressed G protein coupled prostaglandin receptors (Funk 2001).

1.5 tNSAIDs and pdNDSAIDs

Despite their wide use, NSAIDs commonly induce gastrointestinal (GI) adverse effects. It has been estimated that NSAID-induced GI adverse events lead to over 100,000 hospitalizations, US $2 billion in healthcare costs, and 17,000 deaths in the US each year (Frech, Go 2009).

The inhibition of COX-2 activity is primarily driving the antipyretic, analgesic, and anti-inflammatory effects of NSAIDs. COX-1 expression, however, is dominant and constitutive in gastric epithelium and is considered to play an important role in the cytoprotection at this site. Based on this evidence and the assumption that its inhibition would not be critical to NSAID induced enteropathy, purpose designed (pd) NSAIDs, which are selective inhibitors of COX-2 were developed (FitzGerald, Patrono 2001), in

the hope of reducing the undesired GI adverse effects of tNSAIDs, most of which inhibit both COX-1 and COX-2.

Celecoxib and rofecoxib were the first selective COX-2 inhibitors that entered the market. In a randomized double-blinded trial (Simon et al. 1999), the efficacy and incidence of GI complications of celecoxib and naproxen were compared in patients with symptomatic rheumatoid arthritis. It showed that the incidence of endoscopically visualized GI ulcers - a surrogate end point for adverse GI clinical outcomes - in patients receiving celecoxib was significantly lower compared to those receiving naproxen. The Vioxx Gastrointestinal Outcomes Research (VIGOR) trial (Bombardier et al. 2000) was conducted to assess the comparative risk of GI complications from rofecoxib and naproxen. Serious GI complications (perforation, obstruction, and severe upper gastrointestinal bleeding) were 54% lower in rofecoxib group compared to naproxen group. A placebo control group was not included but GI events still occurred in the rofecoxib group. By contrast, the Celebrex Long-term Arthritis Safety Study (CLASS) trial failed to detect a significant difference in the GI complications between celecoxib and diclofenac or ibuprofen when the entire data set was analyzed (Silverstein et al. 2000, Juni, Rutjes & Dieppe 2002). Later, the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET) documented a three to four-fold reduction in ulcer complications in patients treated with lumiracoxib, a COX-2 selective inhibitor, compared to ibuprofen or naproxen (Schnitzer et al. 2004). Results from these last two randomized clinical trials indicate that the distinction in GI outcomes between a tNSAID and a pdNSAID would be likely relative rather than absolute, and that our understanding

of the biology of COX enzymes and the distinct roles of the isoform enzymes are incomplete, raising the importance of further mechanistic investigations.

1.6 NSAID-associated gastrointestinal adverse effects

The mechanism of NSAID-induced GI toxicity is not completely understood, but at least three possibilities have been suggested. First, COX-1 inhibition in gastric epithelial cells reduces the production of mucosal cytoprotective prostaglandins, especially PGI_2 and PGE_2. These prostanoids inhibit gastric acid secretion, enhance mucosal blood flow and promote the secretion of cytoprotective mucus in the intestine. Second, COX-2 inhibition reduces angiogenesis and increases leucocyte adhesion, leading to impaired healing and leucocyte activation and subsequent mucosal injury. Third, NSAIDs cause local irritation from direct contact of orally administered drugs with the mucosa. This allows the acid to diffuse backwards into the mucosa and induces damage at the site (Somasundaram et al. 1995, Rodriguez-Tellez et al. 2001, Abdel-Tawab, Zettl & Schubert-Zsilavecz 2009).

Involvement of intestinal microbiota in NSAID-induced gastrointestinal adverse effect has also been suggested. For example, germ-free rats are resistant to intestinal lesions induced by indomethacin, which is a dual COX inhibitor; this resistance is greatly reduced by associating the animal with a single strain of *E. coli*. (Robert, Asano 1977). Moreover, co-administration of kanamycin, an aminoglycoside antibiotic, alleviates indomethacin-induced intestinal lesions in rats in a dose-dependent fashion (Koga et al. 1999a).

Other studies have also suggested the involvement of intestinal microbiota in the adverse effects of NSAIDs. Kinouchi and colleagues showed that metabolites of *Lactobacillus acidophilus* and *Bifidobacterium adolescentis* inhibit ileal ulcer formation by repressing changes in the intestinal microbiota and lipid peroxidation in rats treated with BFMeT (5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonylphenyl) thiophene) (Kinouchi et al. 1998). Indomethacin treatment also increased microvascular leakage, accompanied by iNOS expression and activity in tissues; pretreatment with antibiotics reduced indomethacin-induced microvascular leakage and iNOS activity (Evans, Whittle 2003).

1.7 Metabolism of NSAIDs

NSAIDs are metabolized in the liver via two major pathways: i) oxidation by cytochrome P450 enzymes (CYPs); and ii) glucuronidation by uridine-5'-diphosphate (UDP)-glucuronosyltransferases (UGTs) (Figure 1.2) (Ulrich, Bigler & Potter 2006).

The major cytochrome P450 enzyme responsible for the oxidation of NSAIDs is CYP2C9 (cytochrome P450, family 2, subfamily C, polypeptide 9) (Tracy et al. 1996, Hamman, Thompson & Hall 1997, Nakajima et al. 1998, Miners et al. 1996), although other P450 enzymes, such as CYP2C8, have also been found to metabolize NSAIDs (Zhao, Leemann & Dayer 1992).

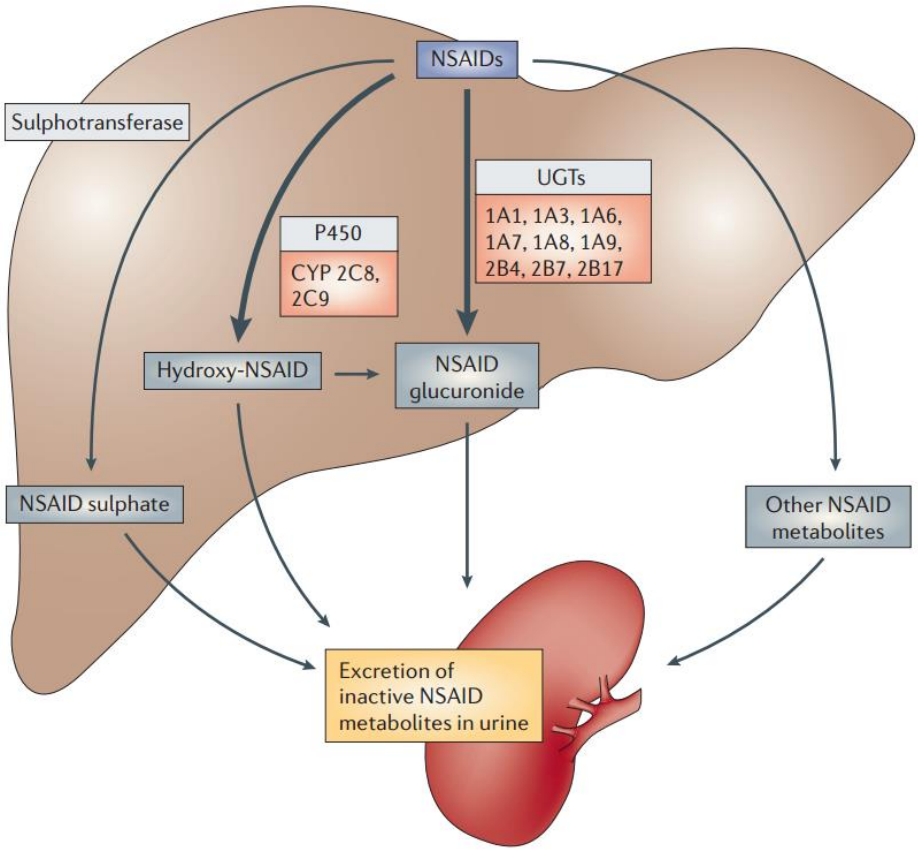

Figure 1.2 NSAID metabolism pathways. The major metabolic pathways include cytochrome P45 enzymes (P450) –mediated oxidation, and uridine-5'-diphosphate (UDP)-glucuronosyltransferases (UGTs) –mediated glururonidation. A minor metabolic pathway includes sulphotransferases-mediated sulphate conjugation. (Adopted from Ulrich CM et al. *Nat.Rev.Cancer.* 2006 Feb; 6(2):130-140).

Hepatic glucuronidation of NSAIDs are mainly catalyzed by multiple UGT enzymes, including UGT1A1, UGT1A9, UGT2B4, and UGT2B7 (Kuehl et al. 2005). UGT1A8 and UGT1A10, which are expressed in intestinal mucosa, have been shown to be involved in

NSAID glucuronidation as well (Cheng, Radominska-Pandya & Tephly 1999, Basu et al. 2004). Some NSAIDs are conjugated with sulfate by sulfotransferases (Falany, Strom & Swedmark 2005, Ulrich, Bigler & Potter 2006).

1.8 Inter-individual variability in NSAID pharmacology

It is often claimed that individuals benefit uniquely from a particular NSAID although this has not been studied systematically (Lee, Goldstein & Pieper 2002). Determining whether and why individuals differ in their responses to NSAIDs are critical steps in the development of personalized approaches to improve efficacy and safety.

One of the likely sources of inter-individual variation of NSAID efficacy is variability in their metabolism through P450 and UGTs enzymes. Both enzyme families are highly polymorphic (Lee, Goldstein & Pieper 2002, Burchell 2003, Fries et al. 2006). For instance, the CYP2C9 L359 variant enzyme showed significantly decreased catalytic activity compared with the wild type enzyme, leading to reduced clearance of NSAIDs (Takanashi et al. 2000, Kirchheiner et al. 2003). Similarly, genetic variation of UGT1A6 also affected its glucuronidation activity compared to the wild type enzyme (Ciotti et al. 1997).

Despite its importance, CYP2C-associated variability can only partially explain the variability amongst individuals in prostanoid inhibition by NSAIDs (Shah 2005, Kirchheiner, Brockmoller 2005, Fries et al. 2006). Considerations should also be given to environmental factors such as nutritional status(Hathcock 1985), the gut microbiota

(Wilson, Nicholson 2009, Clayton et al. 2006), patient age (Roberts, Tumer 1988), disease and the co- or pre-administration of other drugs (Clayton et al. 2006).

1.9 Circadian rhythm

The rotation of the earth results in the oscillation of light during the 24-hour cycle. Organisms adapted to this cycle by developing circadian rhythms based on an endogenous and entrainable mechanism that times daily events such as feeding, temperature, sleep-wakefulness, hormone secretion, and metabolic homeostasis (Saini et al. 2011, Green, Takahashi & Bass 2008). Circadian rhythms are important since they influence drug absorption, distribution, metabolism and excretion, resulting in the circadian variation if pharmacokinetics and pharmacodynamics.

In mammals, this rhythm is controlled by a master clock that resides in the suprachiasmatic nucleus of the hypothalamus. It responds to the changing light cycle and signals this information to peripheral clocks in most tissues (Yang et al. 2013). Circadian rhythms also exist in fungi and cyanobacteria (Hut, Beersma 2011). For example, a pacemaker in cyanobacteria transduces the oscillating daylight signal to regulate gene expression and to time cell division (Johnson 2004, Iwasaki et al. 1999).

The mammalian core clock is regulated tightly by interlocking feedback and feed-forward loops at transcriptional, translational, and post-translational levels (Bass, Takahashi 2010, Xu et al. 2013), as shown in Figure 1.3.

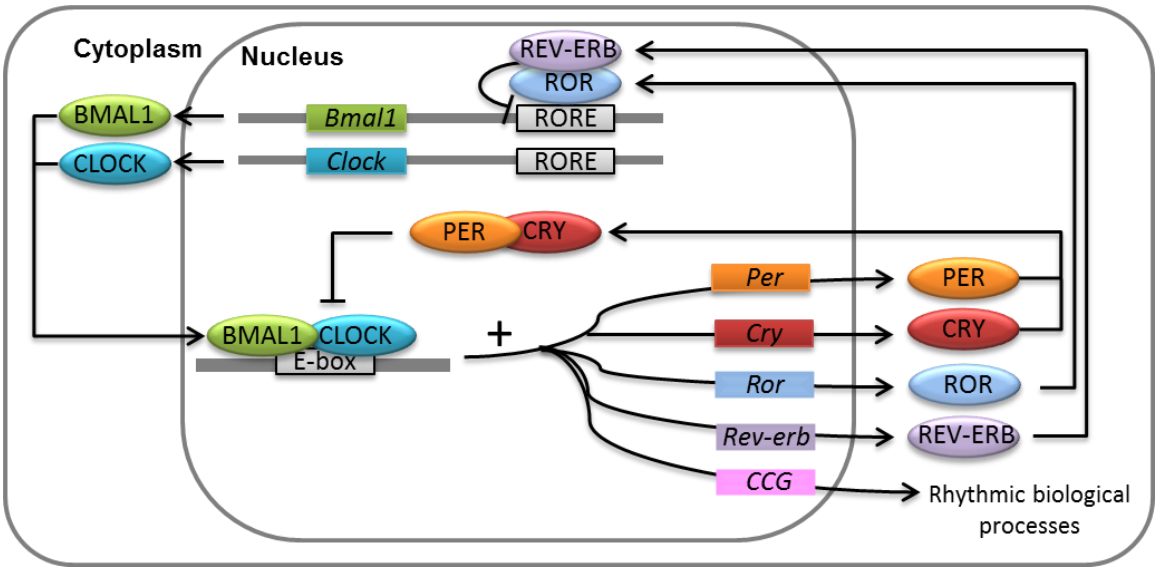

Figure 1.3 The mammalian molecular circadian clock. The BMAL1/CLOCK heterodimer binds to E-box in the promoter regions of *Per* and *Cry* genes to activate their transcription. Once sufficient amounts of PER and CRY have been produced, they dimerize and relocate to the nucleus to inhibit BMAL1/CLOCK activity, hence their own transcription. In addition to this core loop, an interlocking loop was formed by ROR activation and REV-ERB repression of RORE-mediated transcriptions. These feedback loops control the rhythmic expression of clock-controlled genes (CCG) and downstream physiological processes.

The BMAL1 and CLOCK heterodimer binds to the E-box motifs in the promoter regions of *Per* and *Cry* genes, forming the positive limb of the clock. PER and CRY dimerize and relocate to the nucleus, where they repress the activity of BMAL1/CLOCK complex, hence inhibiting the transcription of downstream genes, including their own. This forms

the negative limb of the clock. The retinoic acid receptor (RAR)–related orphan receptor gene (*Ror*) and the nuclear receptor–encoding *Rev-erbα* gene forms additional positive and negative loops.

The core molecular clock exhibits a high degree of redundancy, where compensatory components are up-regulated in response to suppression of one gene in the loop. For example, in the absence of CLOCK, the transcription factor NPAS2 forms a heterodimer with BMAL1 (Baggs et al. 2009). Coincident deletion of both Clock and Npas2 genes is required for suppression of circadian behavior (DeBruyne, Weaver & Reppert 2007). Besides, both *Per* and *Cry* genes have isoforms (*Per1* and *Per2*, *Cry1* and *Cry2*, respectively), and deletion of *Per1* and *Per2* or *Cry1* and *Cry2* is required to ablate the rhythmicity of locomotor activity in mice (Baggs et al. 2009).

In addition to the master clock residing in the suprachiasmatic nucleus, peripheral clocks exist in most tissues (Mohawk, Green & Takahashi 2012). The peripheral clocks not only respond to diverse hormonal and neuronal cues from the master clock, but also have autonomous behaviors. More recently, the peripheral clock in adipocytes has been shown to influence central feeding behavior by modulating lipid signaling to the feeding centers of the brain (Paschos et al. 2012).

Besides the clock networks described in Figure 1.3, a non-transcriptional rhythm also exists. For example, self-sustainable oscillation of KaiC phosphorylation has been established in a test tube by incubating KaiC with KaiA, KaiB, and adenosine triphosphate (Nakajima et al. 2005).Although this is a quite distinct clock, peroxiredoxins,

highly conserved antioxidant proteins, have been shown to undergo redox oscillations in anucleate human red blood cells (O'Neill, Reddy 2011) and in algae, such as *Ostreococcus tauri* , in both cases independently of transcription (O'Neill et al. 2011). Recently, the KaiA, KaiB, and KaiC oscillator has been successfully transplanted to the noncircadian bacterium *Escherichia coli* (Chen et al. 2015).

The circadian clock network is fundamental to maintaining many physiological processes, especially metabolism and immune function. Disruption of this system is associated with various disease phenotypes in mice, such as obesity, diabetes, atherosclerosis, cancer, neurodegeneration, and aging (Yang et al. 2013).

1.10 Chronopharmacology

It has long been known that systemic drug exposure varies with time of administration of the same doses. For example, amikacin, an aminoglycoside antibiotic used to treat different types of bacterial infections, shows significantly higher steady state levels when treated in the early morning hours than in evening (Elting et al. 1990). These changes during different time-of-day may be attributable to circadian rhythms.

The absorption of nitrates (Scheidel, Lemmer 1991), benzodiazepines (Nakano et al. 1984, Muller et al. 1987), calcium channel blockers (Lemmer et al. 1991), and acetaminophen (Kamali, Fry & Bell 1987) is more rapid when administered in the morning rather than in the evening. The concentration of acetaminophen glucuronide in urine during the first 4 hours after drug administration in the morning was found to be twice that when it was administered in the evening (Shively, Vesell 1975). The excretion

processes of drugs vary during different time of day, such as sulfonamides (Dettli, Spring 1967). This may be explained by the temporal variation of urinary pH values.

The cytochrome P450 monooxygenase system is mainly responsible for oxidation of drugs. The expression of cytochrome P450 has been shown to exhibit circadian rhythmicity (Panda et al. 2002). Consistently, the protein levels of cytochrome P450 enzymes also show circadian rhythmicity (Lavery et al. 1999). In addition, mRNA of enzymes that are involved in the cytochrome P450 catalysis system also oscillates, including *Alas1*, which encodes the rate-limiting enzyme in heme biosynthesis (Panda et al. 2002), as well as the P450 oxidoreductase gene, which provides the electrons required for cytochrome P450-mediated monooxygenase reactions (Gachon et al. 2006).

The interaction between the drug and its target is also subject to circadian variation. For example, components of the renin-angiotensin system, which plays an important role in the homeostasis of blood pressure, exhibit significant diurnal variation, with higher plasma renin activity during the night (Brandenberger et al. 1994). Indeed, molecules targeting this system are more effective when administered in the rest phase, such as angiotensin-converting enzyme inhibitor and an angiotensin II AT_1-receptor antagonist (Schnecko, Witte & Lemmer 1995). Recently, a study revealed that almost 175 drugs target oscillating genes (Zhang et al. 2014c). Strikingly, this includes 56 of the top 100 best-selling drugs in the United States (Zhang et al. 2014c).

It is then expected that circadian timing of drug administration plays an important role in the effectiveness and safety of drugs. For example, the tolerance of 5-fluorouracil (5-FU),

an anti-cancer chemotherapy drug, has been shown to vary when delivered at different times of day (Bressolle et al. 1999), possibly due to the diurnal variation in the activities of enzymes involved in 5-FU metabolism and pharmacology, such as thymidylate synthase and dihydropyrimidine dehydrogenase (Harris et al. 1990). Indeed, clinical trials with a chronomodulated drug delivery paradigm showed increased responses and tolerability of 5-FU, leucovorin, and oxaliplatin compared to fixed-infusion-rate delivery (Levi et al. 1994, Levi, Zidani & Misset 1997).

Chronomodulation of drug delivery may also expand the therapeutic index of drugs. For example, since 5-FU tolerability was significantly increased as a result of chronotherapy, an increase in dosing of 5-FU and in the frequency of administration in patients with metastatic colorectal cancer is allowed. As shown by another two trials, improved objective response rates and survival rates were achieved with this approach (Bertheault-Cvitkovic et al. 1996, Levi et al. 1999).

Despite the accumulated evidence suggesting benefits of chronomodulation in drug administration, clinical practice outside of cancer chemotherapy has been little influenced by the accumulation of such information (Abolmaali et al. 2009, Paschos et al. 2010).

1.11 Scope of thesis research

Despite a growing appreciation of the importance of the gut microbiome, few studies have addressed its importance in NSAID pharmacology and its potential relevance to their GI adverse effects. The main objective of my thesis research is to explore the

possibility of a bidirectional interaction between the intestinal microbiota and indomethacin using a mouse model.

Indomethacin, a tNSAID that inhibits both COX-1 and COX-2 enzymes at therapeutic doses (Gierse et al. 1996), has been used to reduce fever, pain, stiffness, and swelling. It evokes gastrointestinal lesions in both humans as well as in experimental animal models (Brodie et al. 1970, Robert, Asano 1977, Stewart et al. 1980, Koga et al. 1999a). We selected indomethacin as a model drug with which to probe this interaction for the following reasons. i) The pharmacology of indomethacin as a mixed inhibitor of COX-1 and COX-2 enzymes is well characterized in model systems and in humans. If an effect was detected, then we could proceed to selective inhibitors of COX-1 and COX-2 to characterize this interaction further. ii) Coincident inhibition/deletion of COX-1 and COX-2 is necessary to evoke GI lesions in mice and both tNSAIDs and pdNSAIDs cause GI lesions in humans. iii) Initial evidence exists showing that indomethacin induced compositional changes in the intestinal microbiota. iv) Indomethacin metabolism undergoes glucuronidation and involves enterohepatic recirculation, where the intestinal microbiota has been shown to play a role.

The first aim of my thesis is to evaluate the composition of the luminal and tissue-associated microbiota along the intestine in mice at baseline as background knowledge. The biomass, diversity, and composition of microbial communities were analyzed for both luminal contents and the mucosal tissues at the proximal, middle, and distal small intestine, the tip of cecum, as well as the proximal, middle, and distal large intestine.

The second aim is to investigate the influence of oral indomethacin administration on the intestinal microbiota composition along the intestinal tract. The biomass, diversity, and composition of microbial communities were compared cross-sectionally between indomethacin-treated mice and vehicle-treated mice in luminal contents and the mucosal tissues at the proximal, middle, and distal small intestine, the tip of cecum, as well as the proximal, middle, and distal large intestine. The biomass, diversity, and composition of microbial communities in feces were compared in indomethacin-treated mice before and after administration.

The third aim is to determine whether the intestinal microbiota is involved in the metabolism of indomethacin. The pharmacokinetics of indomethacin was studied in mice treated with or without antibiotic cocktail. Glucuronidation of indomethacin was evaluated by collecting urine and feces samples from mice after indomethacin administration following the treatment with or without antibiotic cocktail.

The fourth aim is to explore whether the intestinal microbiota composition in mice oscillates diurnally, by collecting feces from mice every 4 hours for 48 hours. Initial assessment of the influence of functional host circadian rhythm on the microbial oscillation during the light-dark cycle was conducted in mice lacking *Bmal1*, which is one of the major components of the mammalian circadian system. Gender differences were also evaluated in both wild type and *Bmal1* knockout mice.

Chapter 2 Basal microbiota composition in murine intestine

2.1 Introduction

To evaluate the effect of indomethacin on the composition of the intestinal microbiota, it is important to analyze the composition of the luminal and tissue-associated microbiota along the intestine in untreated healthy mice.

The microenvironments of the small intestine, cecum, and large intestine are subject to substantial differences in peristalsis, oxygen, enzymes, bile acids, urea, and others (Savage 1977, Walden, Hentges 1975, Mallory et al. 1973, Hoskins, Boulding 1976, Moreau, Ducluzeau & Raibaud 1976). For a given GI site, luminal content and mucosal tissue also provide differential habitats for bacterial communities given the differences in mucus, oxygen, fluid flow rate, epithelial cells, immune barrier, and other factors (Savage 1977, Albenberg et al. 2014). Yet to our knowledge, there is no comprehensive deep sequencing analysis characterizing the murine intestinal microbiota composition along the GI tract, although analysis has been done in dogs, cattle, and horses (Suchodolski et al. 2005, Frey et al. 2010, Schoster et al. 2013), and studies have investigated modest numbers of different sites in mice (Hoffmann et al. 2009). Therefore, we conducted deep sequencing analysis on the intestinal microbiota composition in mice.

2.2 Materials and Methods

2.2.1 Animals

All C57BL/6 mice were purchased from the Jackson Laboratory and housed in our animal facility for at least 2 weeks before experiments. Male mice 10-14 weeks of age were used for all experiments. All animals were fed at libitum with regular chow diet (5010, LabDiet) for the course of study. Mice were kept under 12-hour light/12-hour dark (LD) cycle, with lights on at 7am and off at 7pm. Experimental protocols were reviewed and approved by the Institute for Animal Care and Use Committee at the University of Pennsylvania.

2.2.2 Sample collection

Twenty C57BL/6 mice were sacrificed and the whole intestine was isolated. Luminal contents and adjacent mucosal tissues at the proximal, middle, and distal regions of the small intestine, at the tip of cecum, as well as at the proximal, middle, and distal regions of the large intestine were isolated. The luminal contents were taken from the intestine (about 0.5 centimeter long) gently to avoid contamination by the mucosa. The adjacent mucosal tissues (about 0.5 centimeter long) were opened and washed in water until nothing visible came off the tissue. All samples were weighed and immediately stored at -80 ℃ for following analysis.

2.2.3 DNA extraction for microbiota composition analysis

DNA was extracted from samples with PSP Spin Stool DNA Plus Kit (Stratec) following the manufacturer's instructions with slight modification. Previously collected samples were thawed on ice, and transferred to Lysing Matrix E tubes (MP Biomedicals) with 1,400 µl of stool stabilizer from the PSP kit. They were then disrupted using the TissueLyser II (Qiagen) for 6 minutes at 30 Hz. Samples were then heated at 95 ℃ for 15 minutes, cooled on ice for 1 minute, and centrifuged at 13,400 g for 1 minute to pellet the debris. The supernatant was then transferred to the PSP InviAdsorb tubes, added with 400 µl of stool stabilizer, vortexed for 15 seconds, incubated at room temperature for 1 minute, and centrifuged at 16,000 g for 3 minutes. The supernatant was transferred to a new 1.5 mL receiver tube and centrifuged at 16,000 g for 3 minutes. 1 ml of the supernatant was transferred to a 2 mL safe-lock eppendorf tube containing 31.25 µl of Prot K and was incubated at 70°C for 10 minutes. 500 µl of Binding Buffer P was added and mixed by pipetting up and down. Then 700 µl of the mixture was transferred to RTA spin filter, incubated at room temperature for 1 minute, and centrifuged at 9,300 g for 2 minutes. The filtrate was discarded and the spin filter was loaded with the rest of the mixture. Incubation and centrifuge was repeated, and the RTF filter was transferred to a new 2mL RTA Receiver Tube. Then the filter was loaded with 500 µl of Wash Buffer I, spun down at 9,300 g for 1 minute, and transferred to a new 2 mL RTA Receiver Tube. The filter was then loaded with 700 µl of Wash Buffer II and spun down at 9,300 g for 1 minute. The filtrate was discarded. The filter was then returned to the receiver tube, spun at 16,000 g for 3 minutes to remove EtOH traces, and transferred to a new 1.5 mL

Receiver Tube pre-labeled with sample names. The following elution was conducted with adding 40 µl preheated (70°C) Elution Buffer D, incubation at room temperature for 1 minute, and centrifuge at 9,300 g for 1 minute. A second elution was repeated with 30 µl preheated (70 ℃) Elution Buffer D to maximize the extraction efficiency. Extracted DNA was quantified using NanoDrop 1000 (Thermo Scientific) and stored at -20 ℃ for future use.

Every DNA extraction included a negative extraction control in which water was used instead of fecal pellets. All controls went through the same DNA extraction process as well as following amplification and sequencing processes.

2.2.4 16S rRNA quantification

Quantification of 16S rDNA was performed by real-time PCR using Taqman in triplicate reactions with 10 ng of DNA per reaction. Degenerate bacterial 16S rDNA-specific primers were used for amplification and their sequences were as follows: forward primer, 5’-AGAGTTTGATCCTGGCTCAG-3; reverse primer, 5’-CTGCTGCCTYCCGTA-3’; probe: 5' - /56-FAM/TAA +CA+C ATG +CA+A GT+C GA/3BHQ_1/ - 3', + precedes the position of LNA base.

Quantitative PCR was done on a 7900HT Real-Time PCR System (Applied Biosystems). Thermocycling was performed as follows: initiation at 95 ℃ for 5 minutes followed by 40 cycles of 94 ℃ × 30 seconds, 50 ℃ × 30 seconds, and 72 ℃ × 30 seconds. Signals were collected during the elongation step at 72 ℃.

A standard curve prepared from a near full length clone of Escherichi coli 16S inserted into a Topo Vector was used for normalization for each run of real-time PCR.

2.2.5 V1-V2 16S rRNA region amplification and sequencing

100 ng of DNA was amplified with barcoded primers annealing to the V1-V2 region of the 16S rRNA using AccuPrime Taq DNA Polymerase System with Buffer 2 (Life Technologies). PCR reactions were performed on a thermocycler using the following conditions: initiation at 95 ℃ for 5 minutes followed by 20 cycles of 95 ℃ × 30 seconds, 56 ℃ × 30 seconds, and 72 ℃ × 1 minute 30 seconds, then a final extension step at 72 ℃ for 8 minutes. Each sample was amplified in quadruplicate.

Amplicon purification was conducted with Agencourt AMPure XP beads (Beckman Coulter). 22 μl of each quadruplicate for each sample were pooled into a single eppendorf tube, resulting 88 μl of each sample. Each tube was added with 88 μl of AMPure Beads, votexted for 30 seconds, quickly centrifuged to remove the liquid from cap of tube, and placed in magnetic plate. After a 5-minute incubation at room temperature, the material was gently washed with 200 μl 70% ethanol over beads, and remove the supernatant. There was then a repeat ethanol wash, and incubation at 37 ℃ for 10 minutes with the tubes open. Then the tubes were removed from the magnetic plate, and 50 μl of water was added. Then tubes were vortexed, quickly centrifuged, and placed back on the magnetic holder for 10-minute incubation. The water was pipetted into new pre-labeled eppendorf tube and stored at -20 ℃.

Purified amplicon DNA samples were then sequenced using the 454/Roche GS FLX Titanium chemistry (454 Life Sciences).

2.2.6 16S rRNA sequencing analysis and bioinformatics

Sequence data were processed with QIIME v 1.8.0 (Caporaso et al. 2010b) using default parameters, as shown in Figure 2.1.

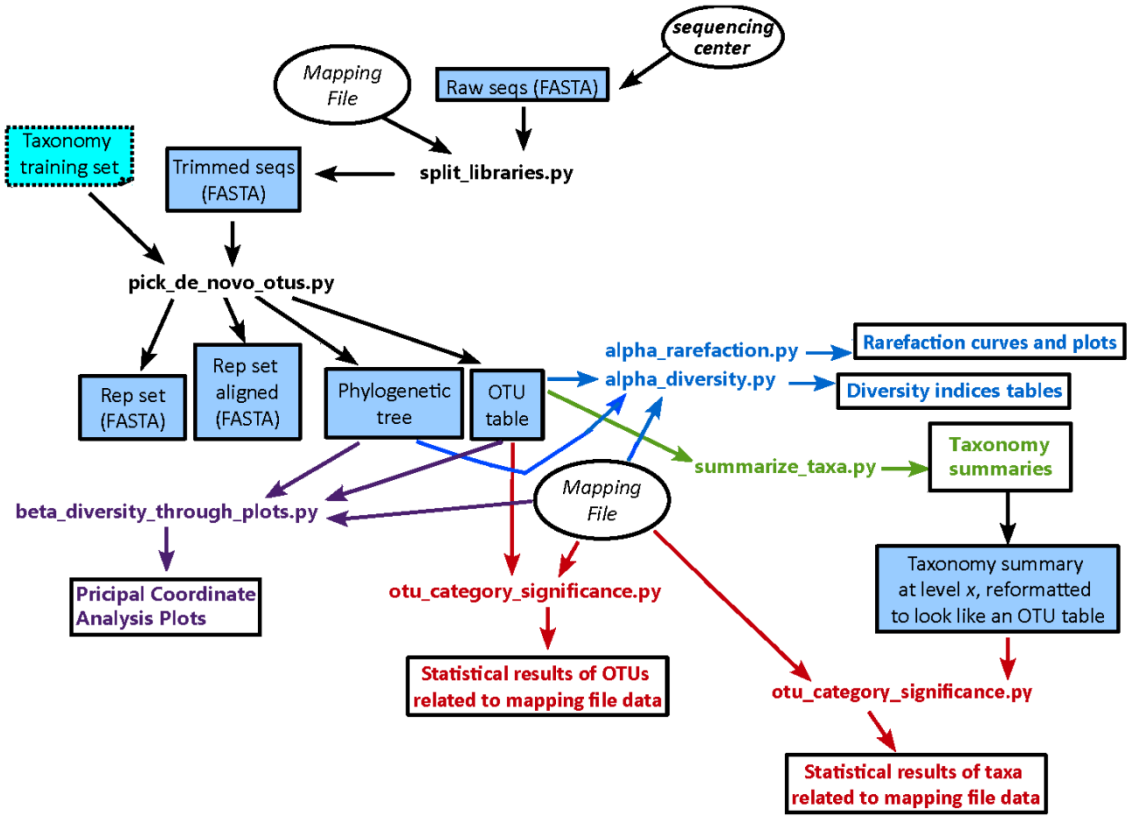

Figure 2.1 QIIME analysis pipeline.

Firstly, samples with less than 200 counts were removed from further analysis. Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity and then assigned taxonomy using the uclust consensus taxonomy classifier. Sequences were aligned using PyNAST (Caporaso et al. 2010a) and a phylogenetic tree was constructed using FastTree (Price, Dehal & Arkin 2009).

Weighted and unweighted UniFrac (Lozupone, Knight 2005) distances were calculated for each pair of samples for assessment of community similarity and generation of principal coordinate analysis (PCoA) plots. Taxonomic composition and alpha diversity were generated for each sample. To compare bacterial abundances across sample groups, group_significance.py was used with default parameters.

2.2.7 PICRUSt analysis

To estimate the functional profile for each microbiota sample, the reads were analyzed with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) version 1.0.0 (Langille et al. 2013). Closed reference OTUs were picked with QIIME from fasta files containing sequences of all samples, and corrected for multiple 16S copy number using "Normalize by Copy Number" in PICRUSt analysis. The normalized OUT table was then used to generate a metagenome of KEGG (Kyoto Encylopedia of Genes and Genomes) ortholog abundances for each sample (Ogata et al. 1999). Predicted metagenomes were collapsed into pathways, and analyzed with STAMP (Parks, Beiko 2010).

2.3 Results

2.3.1 Geographic heterogeneity in the composition of the murine intestinal microbiota

The intestinal microbiota composition is heterogeneous along the intestine as well as between luminal content and mucosal-associated tissue, as shown in Figure 2.2. The luminal content communities (square) form distinct clusters from mucosal tissue communities (triangle) using both unweighted UniFrac distances (describing the bacterial lineages presented in samples) and weighted UniFrac distances (describing the proportions of presented bacterial lineages in samples). This separation is more profound for mucosal-associated tissue.

For both luminal contents and mucosal-associated tissues, microbiota composition in the small intestine has greater inter-mouse variation than that in the cecum or large intestine. As shown in Figure 2.2, small intestinal samples formed disperse clusters, indicating that there is greater inter-sample variability. By contrast, large intestinal samples are tightly clustered together. This is reflected in the heat maps showing the composition at GI sites as well in Figure 2.3.

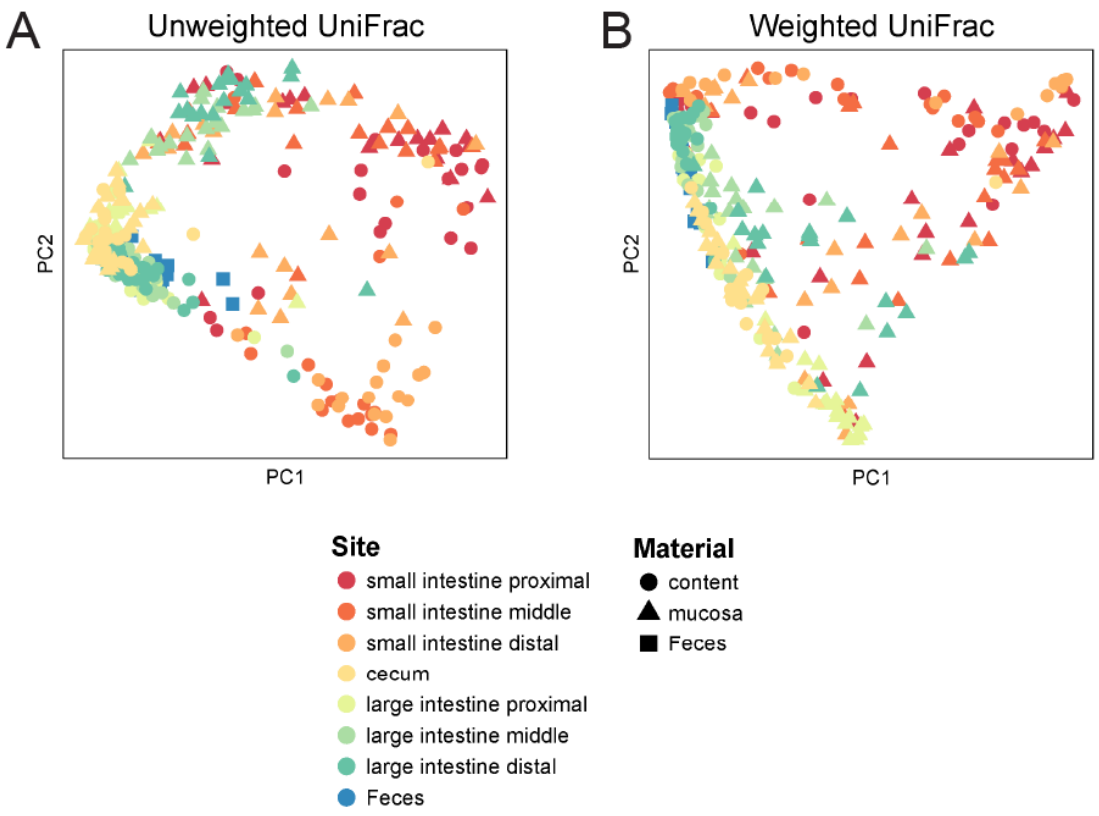

Figure 2.2 Geographic heterogeneity of basal intestinal microbiota composition along the intestine in mice. Principal coordinate analysis (PCoA) of **(A)** unweighted UniFrac and **(B)** weighted UniFrac distances, depicting the comparison of microbial communities from luminal content (round), mucosal tissue (triangle), or feces (square). The base line microbiota compositions along the intestine are heterogeneous at anatomical sites. Each point represents a sample, and each sample is colored according to the habitat sites in the intestine. N=17-20.

The fecal microbiota composition and large intestine luminal microbiota composition share great similarity. In the PCoA plots (Figure 2.2), clusters of fecal samples and those

of large intestine content samples are overlapping with each other, indicating a high similarity amongst such samples, both structurally and proportionally.

2.3.2 Microbiota composition in the luminal content varies along the intestine

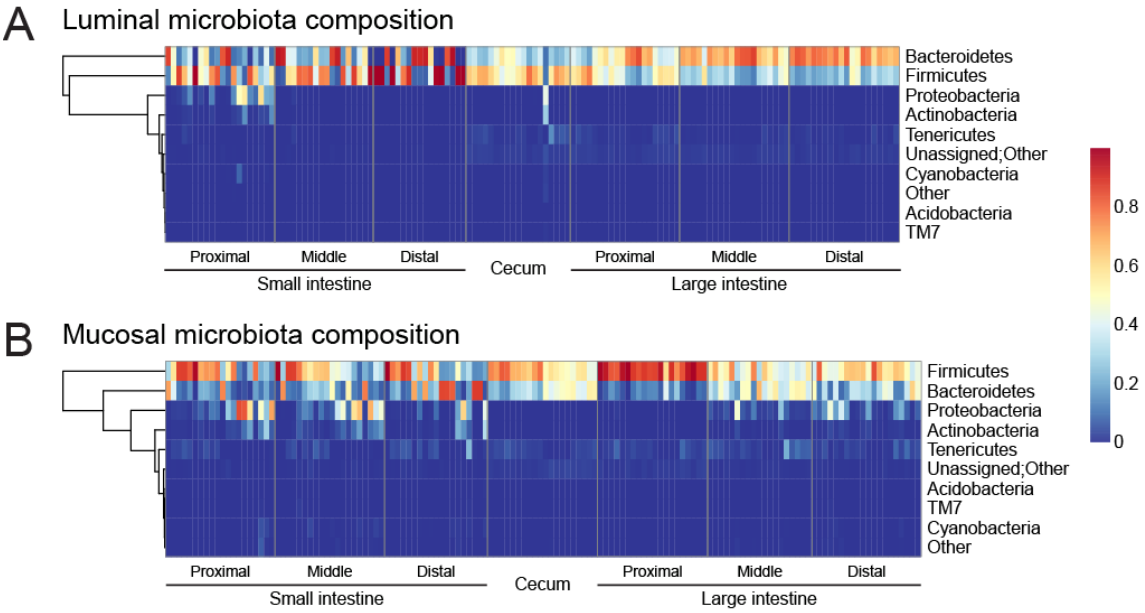

Figure 2.3 Heat map of the microbiota composition in (A) luminal content and (B) mucosal tissue along the intestine. Each column represents one sample, and each row represents one phylum. The proportions of phyla are indicated by the color code to the right. Anatomical sites of the intestine are indicated at the bottom. N=17-20.

In the luminal contents along the intestinal tract, Bacteroidetes and Firmicutes are the dominant phyla throughout the intestine (Figure 2.3 A). Firmicutes is more abundant in the cecum, whereas Bacteroidetes is the dominant population in the large intestine. In the

small intestine, however, the dominance of Bacteroidetes and Firmicutes vary among individuals.

2.3.3 Microbiota composition in the mucosa-associated tissue varies along the intestine

In the mucosa-associated tissues along the intestinal tract, Bacteroidetes and Firmicutes are the dominant phyla throughout the intestine, although other phyla, such as Proteobacteria, Actinobacteria, and Tenericutes, are also present in some regions (Figure 2.3 B). Fimircutes are most abundant in the large intestine proximal region, and are also very abundant in the cecum and in the distal large intestine.

In the small intestine, bacterial lineages of Firmicutes and Proteobacteria are presented, yet higher inter-individual variability in microbiota composition was evident.

2.3.4 The predicted functions of luminal and mucosal microbiotas are different

In addition to the microbiota composition, we also analyzed the predicted gene representations at different anatomical sites, using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). This computational approach predicts the functional composition of microbiome using phylogeny data generated by 16S rRNA sequencing and a public database of reference genomes (Langille et al. 2013). Predicted metagenome of KEGG ortholog abundances for each sample were generated and compared between the luminal content and mucosal tissue along the GI tract. The derived p-values are shown in the heat map (Figure 2.4).


Enzyme Families
Cellular Processes and Signaling
Nucleotide Metabolism
Neurodegenerative Diseases
Xenobiotics Biodegradation and Metabolism
Cell Communication
Sensory System
Nervous System
Metabolism of Terpenoids and Polyketides
Amino Acid Metabolism
Metabolism of Cofactors and Vitamins
Digestive System
Transport and Catabolism
Cell Growth and Death
Metabolism of Other Amino Acids
Cancers
Immune System Diseases
Infectious Diseases
Cardiovascular Diseases
Circulatory System
Signal Transduction
Cell Motility
Replication and Repair
Carbohydrate Metabolism
Signaling Molecules and Interaction
Translation
Genetic Information Processing
Immune System
Folding, Sorting and Degradation
Metabolic Diseases
Metabolism
Poorly Characterized
Lipid Metabolism
Endocrine System
Energy Metabolism
Environmental Adaptation
Membrane Transport
Transcription
Excretory System
Biosynthesis of Other Secondary Metabolites
Glycan Biosynthesis and Metabolism
SI_P
SI_M
SI_D
Ce
LI_P
LI_M
LI_D
p-value
4
0.05
0.01
0.001
2.00E−07

Figure 2.4 *p*-value heat map of comparisons of predicted microbial functions between luminal content and mucosal tissue along the intestine. *p*-values of the differences between luminal content and mucosal tissue at each site were depicted in the heat map. Each column represents comparisons at one GI site. KEGG pathways are indicated to the right. The *p*-values are indicated by the color to the right. The metagenomes in the large intestine are more divergent than the small intestine between luminal content and mucosal tissue. The small intestine middle and distal ends show some divergence. SI, small intestine; Ce, cecum; LI, large intestine. P, proximal; M, middle; D, distal.

In addition to the compositional divergence in the luminal content and mucosal tissue microbiota, the gene representations in these communities are also different from each other, as shown in Figure 2.4.

Although luminal and mucosal microbiota compositions in small intestine are more divergent (Figure 2.2), their predicted functions are not as different (Figure 2.4). By contrast, the predicted functions of luminal and mucosal microbiota in the large intestine are significantly different (Figure 2.4), despite much smaller dissimilarity in their composition (Figure 2.2). Not surprisingly, predicted functions of cecal microbiota in both luminal content and mucosal tissue share high similarity (Figure 2.4), given that they clustered closely in compositional analysis (Figure 2.2).

The luminal microbiome in the large intestine is enriched in genes involved in (i) metabolism of amino acids, energy, glycan, cofactors, vitamins, terpenoids, polyketides,

and nucleotides, (ii) signaling molecules and their interaction, (iii) cell growth and death, (iv) genetic information processing pathways including translation, folding, sorting and degradation, replication and repair. The mucosal microbiome in the large intestine is enriched in genes involved in (i) environmental information processing pathways such as membrane transport and signal transduction, (ii) cell motility and environmental adaption, (iii) xenobiotic biodegradation and metabolism.

2.4 Discussion

We showed that the intestinal microbiota composition bears great heterogeneity at different sites along the intestine. At each of these sites luminal and mucosal microbiota composition also vary. Although compositionally less different, the large intestine microbiota function differentially in luminal content versus in mucosal tissue.

Previous studies revealed compositional differences in microbiota along the GI tract in dogs, cattle, and horses (Suchodolski et al. 2005, Frey et al. 2010, Schoster et al. 2013). However, our study is the first one to use deep sequencing technology to show the distinction amongst microbiota at various regions of the GI tract in mice. Collectively, the microbiota composition along the intestine shows great geographic heterogeneity.

The ever-improving sequencing technology has potentiated the blooming of intestinal microbiome studies over the past several years. The majority of the studies, especially those of the human microbiome, collect fecal samples to minimize the level of invasiveness (Kuczynski et al. 2011). However, the mucosa-associated microbiota in the colon are significantly different from those in feces (Zoetendal et al. 2002). Indeed, we

observed a similar pattern in mice by showing the significant separation between large intestine mucosal microbiota and fecal microbiota (Figure 2.2). Nevertheless, as shown in the PCoA plots, bacterial communities in feces share high similarity with the large intestine content. Hence, it is reasonable to use feces as indirectly representative of the colon lumen. However, for studies focusing on the lining of the GI tract, or focusing on different regions of the GI tract, there is little choice but to collect biopsy samples.

Bacterial communities in the small intestine are highly variable among individuals (Figure 2.2). This is at least partially shaped by the dynamic local microenvironment (Savage 1977). For instance, the small intestine sees the food, water, and air constantly coming in, and it has high flow rates of the content.

The genetic enrichment of the large intestine luminal microbiome in metabolism, cell growth and death, and signaling pathways reflects its dynamic interaction with contents containing nutrients and food debris, while the mucosal microbiome is associated with abundant environmental information processing pathways, reflective of its intimacy and communication with host epithelium.

Chapter 3 Indomethacin causes changes in the microbiota composition

3.1 Introduction

Coincidental disruption of both COX enzymes, such as is achieved by indomethacin at therapeutic doses in humans, is necessary to evoke gastrointestinal lesions in experimental animals (Wallace et al. 2000, Tanaka et al. 2001, Sigthorsson et al. 2002, Takeuchi et al. 2010). However, germ-free rats are resistant to indomethacin-induced intestinal lesions, and the resistance was dramatically reduced when these rats were associated with a single strain of *E coli.* (Robert, Asano 1977). Moreover, an independent study showed that pretreatment of rats with antibiotics attenuated indomethacin-induced enteropathy in a dose-dependent fashion (Koga et al. 1999b). These studies indicate that indomethacin-induced gastrointestinal toxicity is bacteria dependent.

Limited information is available as to the impact of NSAIDs on the intestinal microbiota. 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonylphenyl) thiophene (DuP 697), an experimental COX-2 inhibitor, increased the percentage of gram-negative rods from 1.5% to 49.8% in rats (Kinouchi et al. 1998). Indomethacin resulted in a 1000-fold increase of *Enterococcus faecalis* and a relative diminution of segmented filamentous bacteria (SFB) (Dalby et al. 2006). However, one limitation of these studies is that the technology is not sensitive or comprehensive. Besides, no studies have been done in mice, which is a helpful tool for mechanistic studies.

A more recent study in humans showed that the use of NSAIDs may interact with host age to influence the composition of intestinal microbiota (Makivuokko et al. 2010). It was found that the relative abundance of *Roseburia*, *Ruminococcus*, and *Collinsella spp.* were decreased in elderly NSAID users compared to young adults. Limitations of this study include a small sample size, the use of miscellaneous NSAIDs, uncharacterized for their COX selectivity, and a less than comprehensive microbiota analysis.

Collectively, despite initial evidence on the effect of NSAIDs on the composition of the intestinal microbiota, a detailed, well-designed, and comprehensive study using deep sequencing is still needed to elucidate the effect of indomethacin treatment on the composition of intestinal microbiota in mice.

3.2 Materials and Methods

3.2.1 Animals

All C57BL/6 mice were purchased from the Jackson Laboratory and housed in our animal facility for at least 2 weeks before the experiment. Male mice 10-14 weeks of age were used for all experiments. All animals were fed at libitum with a regular chow diet (5010, LabDiet) for the course of study. Mice were kept under a 12-hour light/12-hour dark (LD) cycle, with lights on at 7am and off at 7pm. Experimental protocols were reviewed and approved by the Institute for Animal Care and Use Committee at the University of Pennsylvania.

3.2.2 Study design

At 12pm, non-fasted mice were administered by gavage with 10 mg/Kg indomethacin (in PEG400), or with PEG400, or left untreated. Each group included 20 male mice. To evaluate indomethacin-induced compositional changes in gut microbiota composition, fecal pellets were collected prior to and 6 hours post drug administration. Then mice were sacrificed to sample the luminal contents and adjacent mucosal tissues at the proximal, middle, and distal regions of the small intestine and large intestine, as well as at the tip of cecum. The study design is illustrated in Figure 3.1. All samples were weighed, placed in empty vials, and immediately stored at -80 ℃ for microbiota composition analysis.

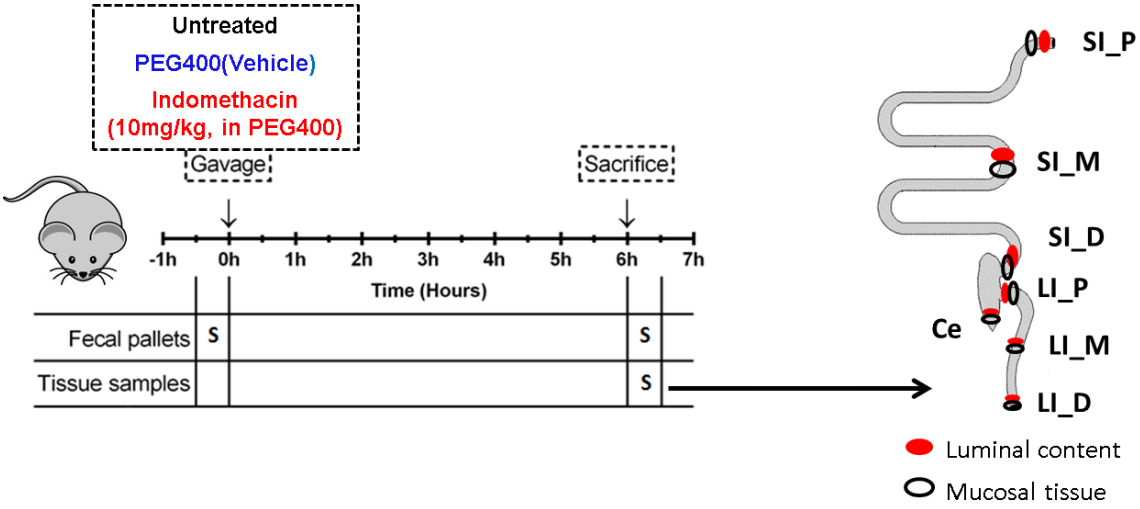

Figure 3.1 The study design of indomethacin-induced compositional changes in murine microbiota.

In the experiment to evaluate indomethacin-induced intestinal damage, mice were sacrificed 24 hours after administration to harvest their small intestines for future histological analysis.

In another experiment to evaluate the inhibitory effect of indomethacin, mice were housed individually in metabolic cages after indomethacin or PEG400 administration for 6 hours to collect their urine and fecal pellets. Each group included 10 mice. Then mice were sacrificed to harvest their intestines. Urinary prostanoid metabolites were determined by mass spectrometric quantitation. Indomethacin concentrations were quantified in urine, feces, and in the luminal contents and mucosal tissues at the proximal, middle, and distal regions of small intestine and large intestine, as well as at the tip of cecum.

3.2.3 Histological analysis of small intestinal damages

Histology of the injured small intestine was analyzed as described (Imaoka et al. 2010). Briefly, small intestine were removed and perfused with phosphate buffered saline (PBS). Tissues were opened along the anti-mesenteric attachment and pinned down for macroscopic examination. The injured segments of the small intestine were trimmed, fixed overnight in 4% (vol/vol) paraformaldehyde at 4 ℃, washed with PBS, and dehydrated with ethanol before embedding in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E) staining.

The degree of inflammation was microscopically graded from 0 to 3 as follows. Grade 0: no signs of inflammation; Grade 1: increased mucosal neutrophils or lymphocytes present;

Grade 2: increased mucosal and submucosal or transmural neutrophils or lymphocytes present; Grade3, mucosal regenerative features with a shortening of villi or ulcers or erosions.

3.2.4 Sample preparation for mass spectrometric analysis of prostanoids

Mouse urine (~100 µl) was spiked with 50 µl mixed stable isotope labelled internal standards. Sample was derivatized with 75 µl methoxime (in HCl) for 15 min at room temperature before solid-phase extraction (SPE).

3.2.5 Sample preparation for mass spectrometric analysis of indomethacin

Tissue and fecal samples were homogenized in 1 ml Millipore H_2O and briefly centrifuged. Samples were added with 2500 ng d_4-indomethacin, vortexed, and incubated at room temperature for 15 min. Samples were centrifuged at 16000 g for 15 min and the upper layer was transferred to a new tube. 100 µl of the supernatant was added with 900 µl H_2O before solid phase extraction (SPE).

Plasma samples (~10 µl) were mixed with 50 µl indomethacin internal standard (300 ng in ACN), 20 µl formic acid and 900 µl H_2O. Samples were vortexed and centrifuged before solid phase extraction (SPE).

3.2.6 Solid-phase extraction

Solid-phase extraction (SPE) was performed according to the Manufacturer's instructions (Strata-X, Phenomenex). Indomethacin and its metabolites were eluted with 1 ml methanol. Prostanoid metabolites were eluted with 1 ml 5% ACN (in ethyl acetate).

3.2.7 Calibration curves for mass spectrometric analysis

To calculate the precise relative amount of indomethacin metabolites, standard curves were prepared in mouse urine. Individual stock solutions of each compound (100 ng/µl) were prepared in ACN and stored at -80 ℃. Working solutions were prepared by mixing equal amounts of corresponding stock solutions and performing serial dilutions with ACN. Seven point calibration samples (0, 0.032, 0.16, 0.8, 4, 20 and 100 ng/µl) for indomethacin were prepared. One large urine sample was obtained from mice without exposure to indomethacin. For each sample, 40 µl of (1 ng/µl) d_4-Indomethacin (Santa Cruz Biotechnology), 10 µl calibration standards were added to 20 mouse urine. The samples were extracted by SPE before LC/MS.

3.2.8 Liquid chromatography/mass spectrometry for indomethacin

Indomethacin was measured using a TSQ Quantum Ultra™ triple quadrupole mass spectrometer (Thermo Scientific) equipped with an ESI ion source. The Mass Spectrometer was connected to a Thermo Scientific Accela HPLC Systems and equipped with a PAL auto sampler and thermocontroller (set at 4 ℃). The CSH C18 Column (2.1 mm X 150 mm, 130Å, 1.7 µm, Waters) was used at a constant 40 ℃. The mobile phase

(A) (90% H_2O/10% (B), 0.2% acetic acid) and mobile (B) (90% ACN/10% methanol) was used at a flow rate of 350 µl/min with a binary gradient (0-12 min, 10-50% B; 12-12.5 min, 50-100% B; 12.5-16 min, 100% B; 16.2-20 min, 10% B). Mass spectrometry was performed in negative mode. The transition for Indomethacin and d_4-Indomethacin are 355.9>311.9 and 359.9>315.9, respectively. Both Q1 and Q3 were operated at 0.7 m/z FWHM. Peak area ratios of target analytes to d_4-Indomethacin internal standards were calculated by Xcalibur Quan software. The data were fitted to the calibration curves to calculate the precise relative amount of indomethacin.

3.2.9 Liquid chromatography/mass spectrometry for prostanoids

Prostanoid metabolites were measured using a Waters Acquity UPLC system comprising a binary pump, an autosampler, and a Xevo TQ-S triple quadrupole mass spectrometer equipped with an electrospray ionization source (Waters Corporation). Chromatographic separation was performed on a Waters UPLC CSH C18 column (2.1 mm X 150 mm, 130Å, 1.7 µm). The UPLC mobile phases consisted of (A) (95%H2O/5% (B), pH=5.7) and (B) 95%ACN/5% methanol. The initial gradient began with 0% B. Mobile phase B increased linearly to 10% at 17 min, to 10.5% at 17.5min, to 11.5% at 32 min, to 20% at 35 min, to 43% at 43 min, to 100% at 43.5 min, and finally go back to 0% at 45.5 min . A 0.35 ml/min flow rate was used throughout the UPLC gradient. The autosampler temperature was set at 4 °C and the UPLC column was heated at 50 ℃. The MS was operated under negative ion mode at MRM mode. The transitions were monitored as previously described (Song et al. 2007). Briefly, systemic production of PGI_2, PGE_2, PGD_2, and TxB_2 was determined by quantifying their major urinary metabolites: 2, 3-

dinor 6-keto $PGF_{1\alpha}$ (PGI-M); 7-hydroxy-5, 11-diketotetranorprostane-1, 16-dioic acid (PGE-M); 11, 15-dioxo-9_{α}-hydroxy-2, 3, 4, 5-tetranorprostan-1, 20-dioic acid (tetranor PGD-M); and 2, 3-dinor TxB_2 (Tx-M), respectively. Results were normalized with creatinine (Oxford Biomedical Research). Peak areas were obtained using MassLynx® software (Waters Corporation).

3.2.10 DNA extraction for microbiota composition analysis

DNA extraction was performed as described in Chapter 2 (2.2.3).

3.2.11 16S rRNA quantification

Quantification of 16S rRNA was performed by real-time PCR as described in Chapter 2 (2.2.4).

3.2.12 V1-V2 16S rRNA region amplification and sequencing

16S rRNA gene amplification was performed as described in Chapter 2 (2.2.5).

3.2.13 Bioinformatics

Sequence data were processed with QIIME v 1.8.0 (Caporaso et al. 2010c) using default parameters, as described in Chapter 2 (2.2.6).

3.3 Results

3.3.1 Mice are systemically and locally exposed to indomethacin

Exposure to indomethacin was measured by mass spectrometry.

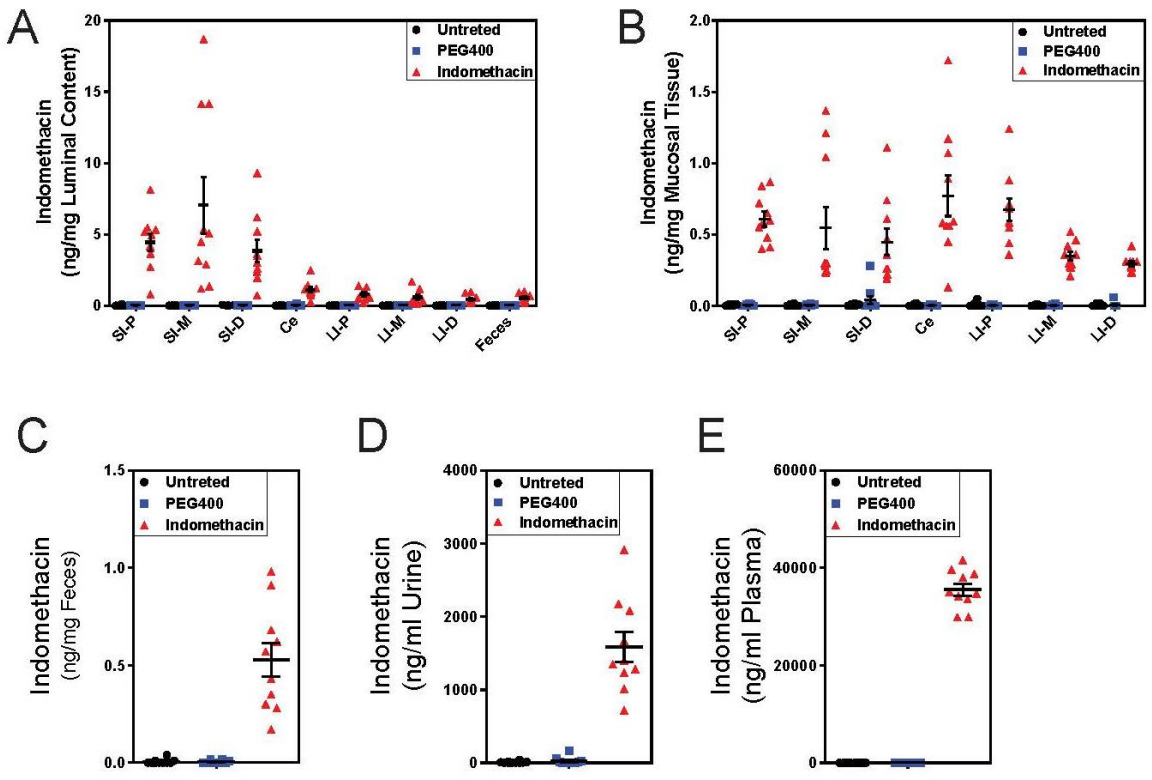

Figure 3.2 C57BL/6 mice are systemically and locally exposed to indomethacin. Indomethacin concentrations were measured in samples and corrected by sample weight. Indomethacin is detected along the intestine in both **(A)** luminal content and **(B)** mucosal tissue in mice of indomethacin (red) group, but not in those of PEG400 (blue) or untreated (black) groups. In **(C)** feces, **(D)** urine, and **(E)** plasma, indomethacin is also detected in mice of indomethacin (red) group, but not in those of PEG400 (blue) or untreated (black) groups. N=10/group. Mean ± S.E.M. shown. SI, small intestine; Ce, cecum; LI, large intestine. P, proximal; M, middle; D, distal.

As shown in Figure 3.2, indomethacin was readily detected, both systemically and locally, in indomethacin-treated mice. The average indomethacin level in plasma was 35.5 μg/ml.

Indomethacin was also detected in urine and feces due to fecal and urinary excretion, with the average level of 1.58 μg/ml and 0.53 ng/mg, respectively. The intestine was fully exposed to indomethacin, forming a gradient from small intestine luminal content (5.1 ng/mg tissue on average), through small intestine mucosa (0.53 ng/mg tissue on average), cecum luminal content (1.11 ng/mg tissue on average) and mucosa (0.77 ng/mg tissue on average) to large intestine luminal content (0.6 ng/mg tissue on average) and mucosa (0.44 ng/mg on average).

3.3.2 Indomethacin inhibits both COX-1 and COX-2 in mice

Eicosanoid biosynthesis was estimated by measuring their corresponding urinary metabolites using mass spectrometry, as shown in Figure 3.3. Compared to untreated and PEG400-treated mice, indomethacin treatment caused 74.6% reduction in the urinary PGD-M (the major PGD_2 metabolite) concentration, 82.9% reduction in the urinary PGE-M (the major PGE_2 metabolite) concentration, 53.1% reduction in the urinary PGI-M (the major PGI_2 metabolite) concentration, and 87.4% reduction in the urinary Tx-M (the major metabolite of TxA_2) concentration, indicating the suppression of prostanoid production by indomethacin. Among these, PGD_2 and TxA_2 are primarily derived from COX-1, whereas PGE_2 and PGI_2 are primarily derived from COX-2. Therefore, both COX-1 and COX-2 were inhibited by 6-hour treatment of a single oral dose of 10 mg/kg indomethacin in mice.

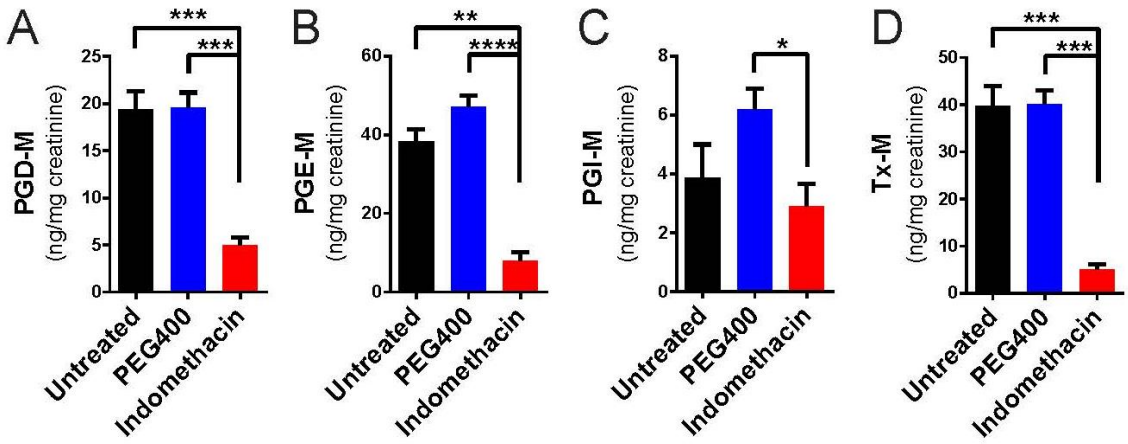

Figure 3.3 Inhibitory effects of indomethacin on COX-1 and COX-2 in C57BL/6 mice. Mice were administered by gavage with or without 10mg/Kg indomethacin (red) or PEG400 (blue) and urine were collected for the analysis of prostanoid metabolites. **(A)** PGD-M, **(B)** PGE-M, **(C)** PGI-M, and **(D)** Tx-M are reduced in indomethacin-treated mice. N=6/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Kruskal-Wallis test, multiplicity adjusted. Mean ± S.E.M. shown.

3.3.3 Indomethacin induces injury in the small intestine

A single oral dose of 10 mg/kg indomethacin induced small intestinal damage in mice within 24 hours of drug administration. Indomethacin induced severe intestinal damage compared to vehicle (PEG400) treatment. Beside, indomethacin mostly caused Grade 3 damage, which includes shortening of villi, ulcers or erosions (Figure 3.4 A). Representative H&E staining sections of Grade 3 injuries were shown in Figure 3.4 B.

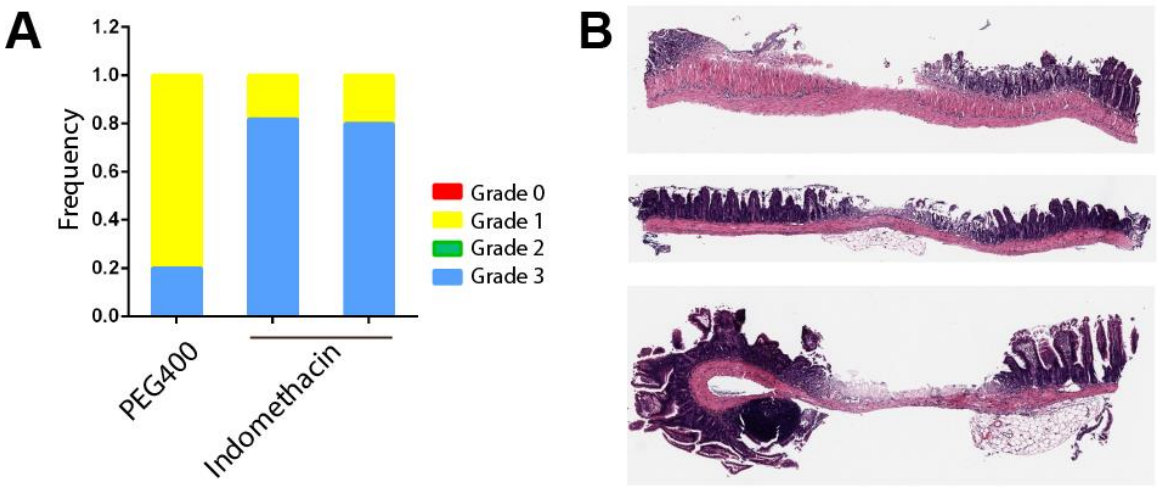

Figure 3.4 Indomethacin induces small intestinal damages in C57BL/6 mice. (A) Frequency of indomethacin induced small intestinal injuries that are categorized into 4 grades. **(B)** Representative sections of hematoxylin and eosin staining of injured small intestine 24 hours after 10 mg/Kg indomethacin treatment.

3.3.4 Bacterial load along the intestine is not altered by indomethacin

Cross-sectional analysis of the luminal contents and mucosal tissues along the intestine showed that indomethacin barely caused changes in16S gene copy numbers, despite a slight decrease in middle large intestine luminal content and proximal large intestine mucosal-associated tissue (Figure 3.5). Therefore, 10 mg/Kg indomethacin treatment had no effect on the bacterial load along the intestine.

A vehicle effect was evident (Figure 3.5). Mice treated with PEG400 showed a decrease in luminal biomasses and an increase in mucosal tissue biomasses in the distal end of large intestine, compared to untreated mice.

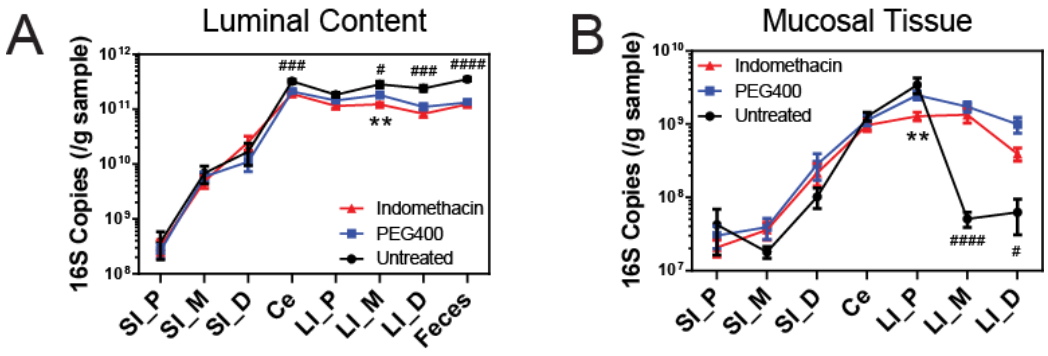

Figure 3.5 Bacterial biomasses at anatomical sites along the intestine. 16S rRNA gene copies per gram of **(A)** luminal contents and **(B)** mucosal tissues at anatomical sites along the intestine in indomethacin (red), PEG400 (blue), and untreated (black) groups. Microbial loads at anatomical sites along the intestine are barely different between PEG400 and indomethacin groups, although PEG400 causes changes by itself. ** $p <$ 0.01 by multiple t test comparing PEG400 versus indomethacin groups, FDR corrected. # $p < 0.05$, ### $p < 0.001$, #### $p < 0.0001$ by multiple t test comparing untreated versus PEG400 groups, FDR corrected. N=20/group. Mean ± S.E.M. shown. SI, small intestine; Ce, cecum; LI, large intestine. P, proximal; M, middle; D, distal.

3.3.5 Alpha diversities are altered in colon

Microbial communities along the intestine were analyzed by evaluating species richness and diversity measured by the Observed Species and Shannon Index. Comparison between the indomethacin and PEG400 groups revealed changes in the large intestine.

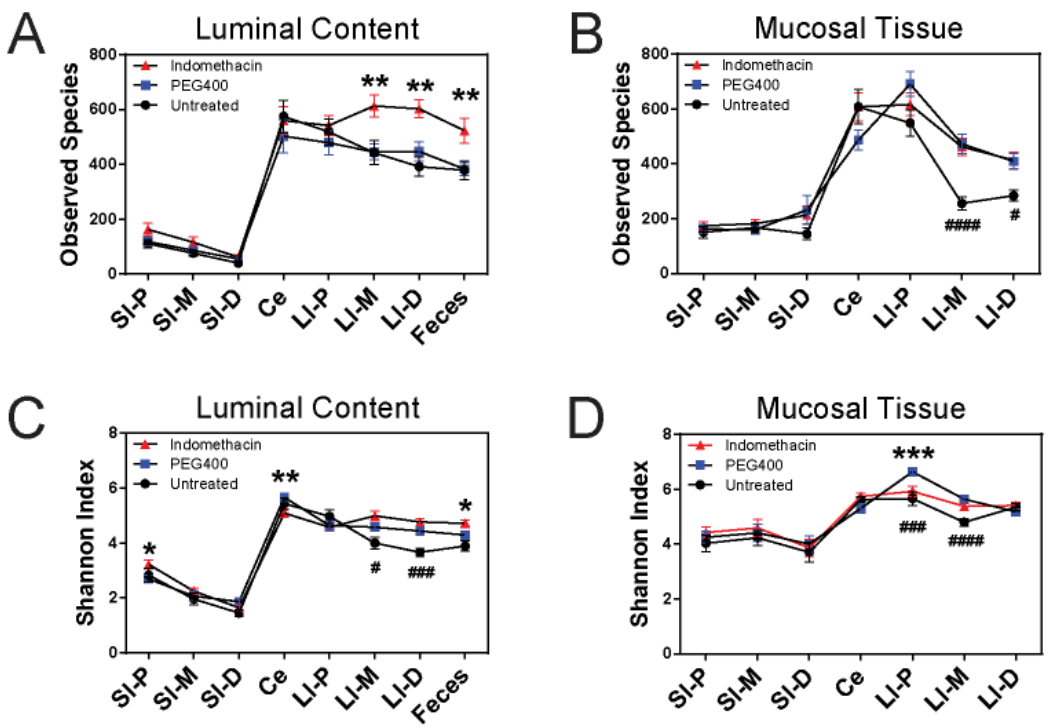

Figure 3.6 Bacterial diversity at anatomical sites along the intestine. (A) Observed Species of luminal content. **(B)** Observed Species of mucosal-associated tissue. **(C)** Shannon Index of luminal content. **(D)** Shannon Index of mucosal-associated tissue. Richness and diversity of microbial communities were shown for indomethacin (red), PEG400 (blue), and untreated (black) groups. Indomethacin altered microbial diversity in the distal intestine, although PEG400 also causes changes in the distal intestine by itself. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by multiple t test comparing PEG400 versus indomethacin groups, FDR corrected. # $p < 0.05$, ### $p < 0.001$, #### $p < 0.0001$ by multiple t test comparing untreated versus PEG400 groups, FDR corrected. N=20/group. SI, small intestine; Ce, cecum; LI, large intestine. P, proximal; M, middle; D, distal.

Indomethacin caused an increase in Observed Species (Figure 3.6 A, B) in the middle and distal large intestine luminal content, as well as in feces, without affecting the mucosal tissues. The Shannon Index (Figure 3.6 C, D) was decreased in the luminal content of the cecum and in the mucosal tissue of proximal large intestine, and increased in feces.

PEG400 alone increased microbial diversity in the distal large intestine. For example, PEG400 induced increase of observed species in the large intestine middle and distal mucosa tissues, increase of Shannon index in the large intestine middle and distal luminal content, as well as increase of Shannon index in the large intestine proximal and middle mucosa tissues.

3.3.6 Indomethacin induces changes in luminal and mucosal microbiota composition

Indomethacin induced compositional changes in intestinal microbiota primarily in the large intestine. Principal coordinate analysis (PCoA) revealed significant separation of bacterial communities between indomethacin- or PEG400-treated mice, as shown in Figure 3.7. Statistics of PCoA was presented in Table 3-1.

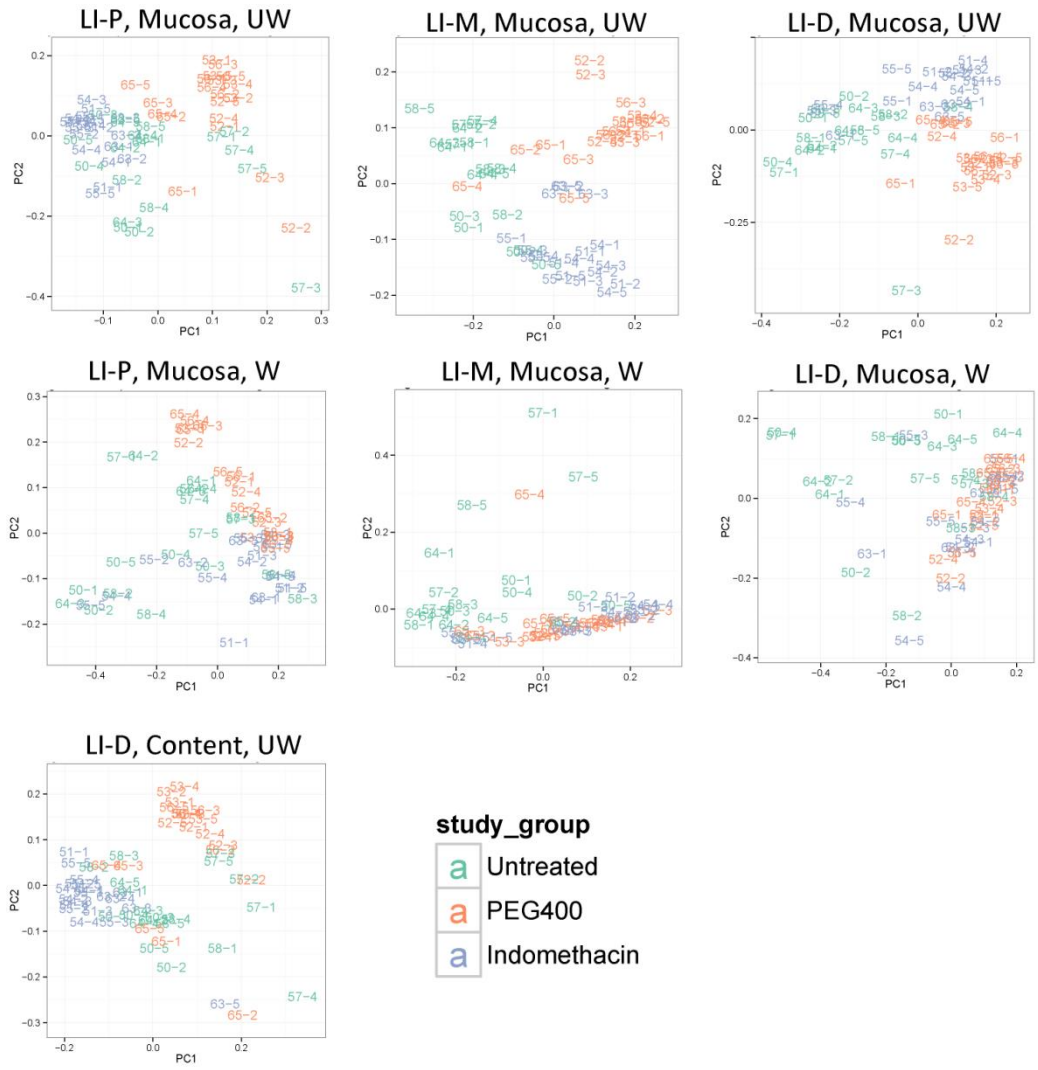

Figure 3.7 Indomethacin-induced compositional changes in the large intestine. Principal coordinate analysis (PCoA) of unweighted UniFrac (UW) and weighted UniFrac (W) distances, depicting the comparison of microbial communities from indomethacin (blue), PEG400 (red), and untreated (green) groups at anatomical sites in large intestine. Each point represents a sample, and each sample is colored according to the habitat sites in the intestine. N=17-20. LI, large intestine. P, proximal; M, middle; D, distal.

Table 3-1 PCoA statistics of indomethacin-induced microbial changes

GI site	Sample type	UniFrac distance type	q-value by PERMANOVA	Pairwise comparison (PEG400 vs. Indomethacin)
Proximal large intestine	Mucosal tissue	Unweighted	0.001	0.009
Proximal large intestine	Mucosal tissue	Weighted	0.001	0.004
Middle large intestine	Mucosal tissue	Unweighted	0.001	0.026
Middle large intestine	Mucosal tissue	Weighted	0.001	0.009
Distal large intestine	Mucosal tissue	Unweighted	0.001	0.006
Distal large intestine	Mucosal tissue	Weighted	0.004	0.009
Distal large intestine	Luminal content	Unweighted	0.002	0.036

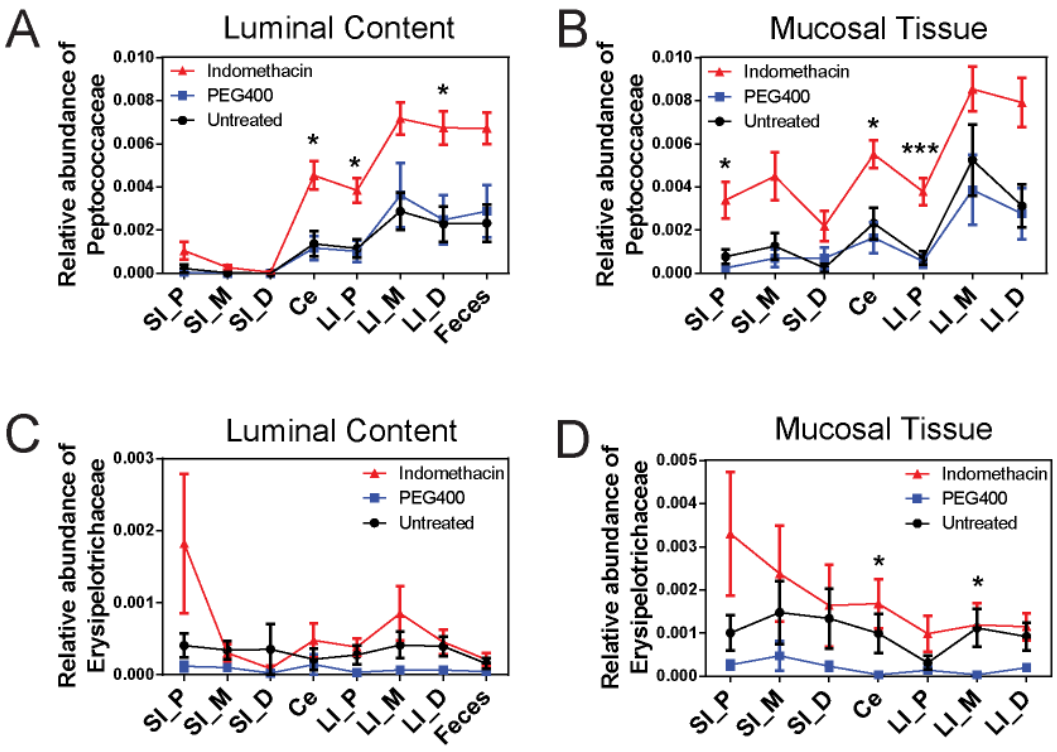

Figure 3.8 Indomethacin-induced changes in bacterial abundances. The relative abundance of **(A)** *Peptococcaceae* in luminal content, **(B)** *Peptococcaceae* in mucosal tissue, **(C)** *Erysipelotrichaceae* in luminal content, and **(D)** *Erysipelotrichaceae* in mucosal tissue at anatomical sites along the intestine are significantly elevated in indomethacin (red) group than in PEG400 (blue) and untreated (black) groups. * $p <$ 0.05, *** $p < 0.001$ by QIIME analysis, FDR corrected. Mean ± S.E.M. shown. SI, small intestine; Ce, cecum; LI, large intestine. P, proximal; M, middle; D, distal.

The abundance of discrete bacterial lineages was also affected by indomethacin. *Peptococcaceae* expanded in the luminal content of cecum, the proximal and distal large intestine, as well as in mucosal tissues of the cecum and the proximal large intestine, as

shown in Figure 3.8 A and B. *Erysipelotrichaceae* expanded in the mucosal tissues of cecum and middle large intestine, yet were less affected in the luminal content, as shown in Figure 3.8 C and D.

3.3.7 Indomethacin has no effect on fecal microbial biomass

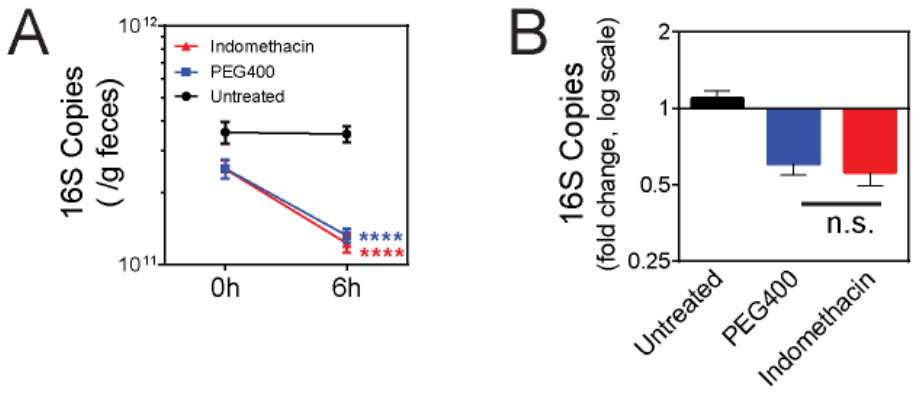

Figure 3.9 Indomethacin has no effect on fecal microbial biomass. (A) 16S rRNA gene copies per gram of feces at 0h and 6h in indomethacin (red), PEG400 (blue), and untreated (black) groups. **(B)** Fold changes of 6h-to-0h 16S rRNA gene copy number in indomethacin (red), PEG400 (blue), and untreated (black) groups. Both PEG400 and indomethacin groups have lower bacterial load at 6h, whereas these is no between-group differences at 0h or 6h. **** $p < 0.0001$ by Mann-Whitney test comparing 0h versus 6h. N=20/group. Mean ± S.E.M. shown.

To address the longitudinal effect of indomethacin on fecal microbiota biomass, 16S rRNA gene copy numbers before and after indomethacin administration were evaluated.

As shown in Figure 3.9 A, both the PEG400 and indomethacin-treated groups showed a significant reduction in 16S rRNA gene copy numbers, indicating a reduced biomass over time. However, statistical analysis revealed no difference between PEG400 and indomethacin groups either before (0h) or after (6h) treatment (Figure 3.9 B), indicating that the reductions in both groups was due to a vehicle effect. Therefore, indomethacin had no effect on fecal microbial biomass.

3.3.8 Indomethacin increases fecal microbial diversity

The longitudinal effect of indomethacin on microbial evenness and diversity was evaluated by measuring Observed species and the Shannon index.

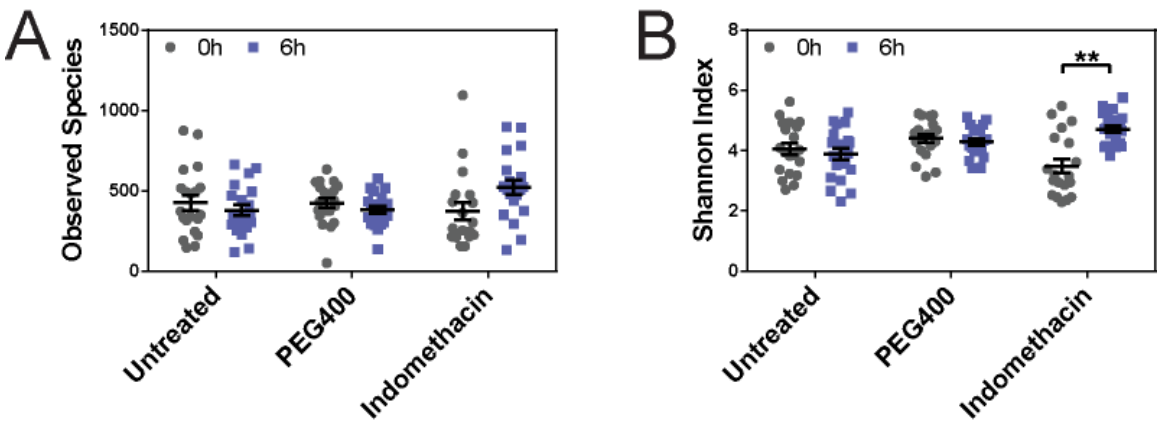

Figure 3.10 Indomethacin increases fecal microbial diversity. (A) Observed Species and **(B)** Shannon Index are increased at 6h in indomethacin-treated mice, while unchanged in Untreated and PEG400 groups. ** $p < 0.01$ by multiple t test, FDR corrected. N=19-20/group. Mean ± S.E.M. shown.

The Shannon index was significantly increased 6 hours after indomethacin treatment, whereas it was unchanged in untreated and PEG400-treated mice (Figure 3.10 A). There was no significant change in Observed species (Figure 3.10 B). Therefore, indomethacin increased fecal microbial diversity 6 hours after administration.

3.3.9 Fecal microbiota composition is altered by indomethacin treatment

Collection of fecal pellets from the same mouse before and after indomethacin treatment revealed within-individual compositional changes (Figure 3.11 A). Fecal microbial communities at 0h and 6h are overlapping with each other in Untreated and PEG400 groups, but formed separated clusters in indomethacin group.

Indomethacin treatment induced a significant decrease of the abundance of Bacteroidetes (Figure 3.11 B) as well as a significant increase of Firmicutes (Figure 3.11 B) in the feces. However, no change occurred in untreated or PEG400-treated mice.

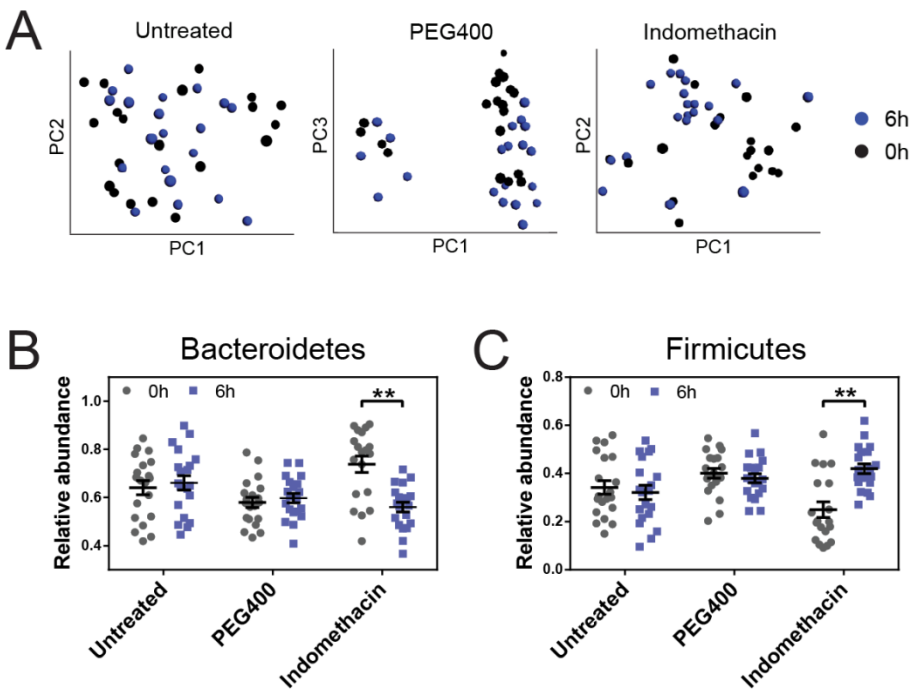

Figure 3.11 Indomethacin induces longitudinal changes in fecal microbiota composition. (A) Principal coordinates analysis (PCoA) of unweighted UniFrac distances was used to compare the fecal microbial communities at 0h (black) versus 6h (blue) of untreated (left), PEG400 (middle), and indomethacin (right) groups. Each point represents a sample. Fecal microbial communities at 0h and 6h are overlapping in untreated and PEG400 groups, whereas they are separated in the indomethacin group. **(B)** The relative abundance of Bacteroidetes is decreased at 6h (blue) after indomethacin treatment. **(C)** The relative abundance of Firmicutes is increased at 6h (blue) after indomethacin treatment. ** $p < 0.01$ by multiple t test, FDR corrected. N=19-20/group. Mean ± S.E.M. shown.

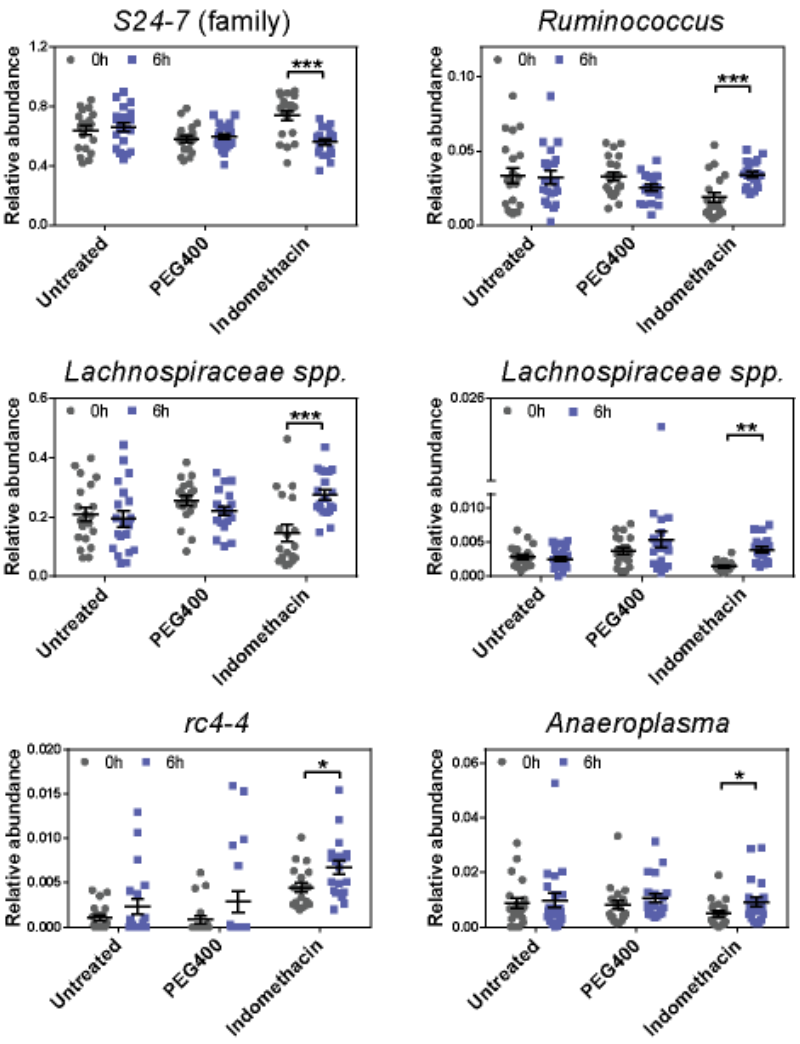

Figure 3.12 Indomethacin alteres genera abundances in fecal microbiota. There was a decrease in the relative abundance of *S24-7* (family), and increases in those of *Ruminococcus*, *Lachnospiraceae sp.*, *Lachnospiraceae sp.*, *rc4-4*, and *Anaeroplasma* at 6h (blue). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by QIIME analysis, FDR corrected. N=19-20/group. Mean ± S.E.M. shown.

Examination at lower taxonomic levels also revealed indomethacin-induced changes in fecal microbiota (Figure 3.12). For example, *S24-7 spp.*, a Bacteroidetes, was decreased significantly in indomethacin-treated mice. In parallel, some Firmicutes were significantly increased in indomethacin-treated mice, including *Ruminococcus*, *Lachnospiraceae*, and *rc4-4*, as were *Anaeroplasma*, a genus of Tenericutes. These taxa were not altered in untreated or PEG400-treated mice.

The abundance of *Clostridiales spp.*, a genus of Firmicutes, was increased in both PEG400- and indomethacin-treated mice at 6h (Figure 3.13 A), consistent with a vehicle effect. By contrast, the abundance of *Ruminococcaceae spp.*, another genus of Firmicutes, was decreased in PEG400-treated mice but increased in indomethacin-treated mice (Figure 3.13 B). This indicates that indomethacin may upregulate this genus, an effect great enough to counteract the downregulation by PEG400. The abundance of *Lactobacillus*, a genus of Firmicutes, was increased significantly in PEG400-treated mice, yet was not altered in untreated or indomethacin-treated mice (Figure 3.13 C, left), Similarly, *Oscillospira*, a genus of Firmicutes, was decreased in PEG400-treated mice only (Figure 3.13 C, right).

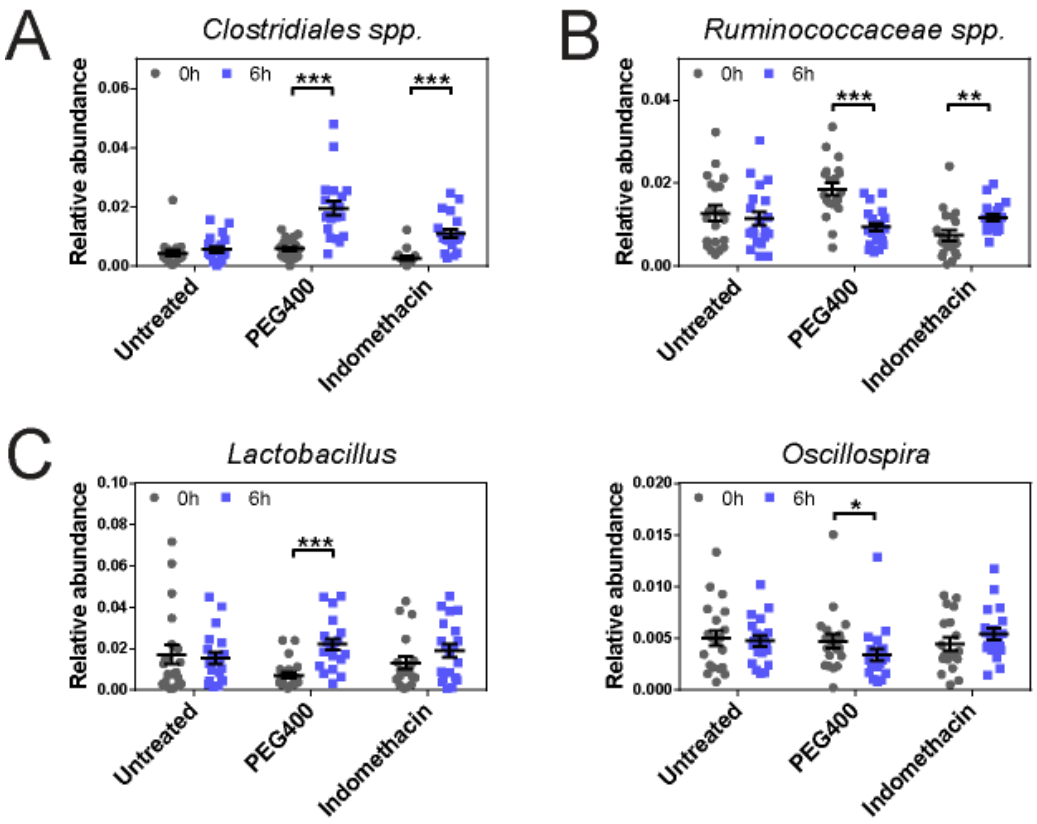

Figure 3.13 Vehicle effect on fecal microbiota composition. (A) The relative abundances of *Clostridiales spp.* is increased in both PEG400 and indomethacin groups at 6h. **(B)** The relative abundance of *Ruminococcaceae spp.* is decreased in PEG400 group but increased in indomethacin group at 6h. **(C)** PEG400 induced an increase of *Lactobacillus* (left) and a decrease of *Oscillospira* (right), whereas there is no change in untreated or indomethacin groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by QIIME analysis, FDR corrested. N=19-20/group. Mean ± S.E.M. shown.

3.4 Discussion

The selected dose and treatment of indomethacin resulted in detectable systemic drug exposure; it was present in luminal content and along the mucosa of the small intestine and large intestine and was measured in plasma, urine and feces. Reflective of its mechanism of analgesic and anti-inflammatory action, this dose of indomethacin suppressed endogenous biosynthesis of prostaglandins derived primarily from COX-1 (PGD_2 and Tx) and COX-2 (PGI_2 and PGE_2) (McAdam et al. 1999, Ricciotti, FitzGerald 2011), as reflected by urinary excretion of their major metabolites. Indomethacin at the dose selected also resulted in small intestinal damage in mice, reminiscent of the enteropathy caused by NSAIDs in humans (Allison et al. 1992, Smale et al. 2001).

We showed that only one single oral dose of indomethacin was sufficient to perturb the intestinal microbiota, specifically within the cecum, large intestine and feces. Indomethacin-induced effects were less evident in the small intestine. We have shown that microbiota composition in the small intestine has higher inter-individual variance (Figure 2.2), which may limit the ability to detect the impact of interventions. It is possible that (i) indomethacin has no effect in small intestine at all due to the rapid turnover in the local environment; (ii) indomethacin-induced changes are too small to overcome the inter-individual differences for detection. Therefore, impacts in the small intestine may remain difficult to interpret for now.

Indomethacin induced expansion of *Peptococcaceae* and *Erysipelotrichaceae* in the large intestine. *Peptococcaceae* are Gram-positive anaerobic bacteria of Firmicutes.

Environmental cultures of *Peptococcaceae* originated from soil and groundwater have been shown to mediate anaerobic benzene biodegradation (van der Zaan et al. 2012, Luo et al. 2014, Cupples 2011), and its abundance is significantly increased when grown on benzene (Luo et al. 2014). It is hence possible that exogenous indomethacin provides an extra substrate and promoted the expansion of *Peptococcaceae* in the GI tract. The physiological role of *Peptococcaceae* is poorly understood, although it is commonly found in the mouth and intestinal and respiratory tracts of human and other animals (Rogosa 1971). *Erysipelotrichaceae* is a Gram-positive bacterial family of Firmicutes. It has been shown to be positively associated parenteral nutrition associated liver injury by activating TLR2 signaling pathways (Harris et al. 2014). More recently, blooming of *Erysipelotrichaceae* has also been associated with obesity (Zhang et al. 2009), colorectal cancer (Zhu et al. 2014, Chen et al. 2012), and Crohn's disease (Kaakoush et al. 2015). Elevation of these pro-inflammatory bacteria in the intestine, together with small intestinal damage developed in a later stage (Figure 3.4) may provide a link between indomethacin-induced compositional changes along the GI tract and the GI toxicity of indomethacin.

Indomethacin also induced pro-inflammatory shifts in the composition of fecal microbiota, for example, a significantly increased ratio of Firmicutes to Bacteroidetes. This shift has been previously reported in genetically obese mice (Ley et al. 2005, Turnbaugh et al. 2006), obese children (Bervoets et al. 2013), and obese adults (Ley et al. 2006b). Besides, the decrease of *S24-7*, a family of Bacteroidetes, such as induced here by indomethacin, has been observed in a mouse model of colorectal cancer (Liang et al.

2014), as well as in mice with high fat diet-induced obesity (Evans et al. 2014). *Lachnospiraceae,* also upregulated by indomethacin, have been associated with lupus (Zhang et al. 2014a), drug-induced liver toxicity (Xu et al. 2015). It also contributes to the development of obesity and diabetes in genetically susceptible mice (Kameyama, Itoh 2014).

The indomethacin vehicle, PEG400, was found to have an effect on microbial diversity and composition (Harrell et al. 2012). In the distal intestine, PEG400 caused decreased diversity in luminal content and increased diversity in mucosal tissue; the abundances of fecal *Clostridiales spp.* and *Lactobacillus* were increased while the abundance of *Oscillospira* was decreased (Figure 3.13). Possible explanations for the vehicle effect include a water-absorbing dilutional effect and/or a "flushing" effect carrying upstream luminal bacteria down to the large intestine. Similarly, Golytely (PEG 3350) has been previously reported to cause changes in the mucosal-associated microbiota in the human colon (Harrell et al. 2012). The trial design, however, allowed us detect indomethacin effects independent of vehicle effects.

Chapter 4 The intestinal microbiota affects the pharmacokinetics of indomethacin

4.1 Introduction

NSAIDs show considerable inter-individual variation in pharmacokinetics in humans (Brune 1985, Brune 1987). This may contribute significantly to the observed inter-individual differences in the efficacy and risk of GI complications from indomethacin. Deciphering the sources of such variability is a critical step in developing a more personalized approach to using these drugs more safely and efficaciously.

Intestinal bacteria have been shown to mediate the biotransformation of over 30 approved drugs by directly or indirectly influencing their metabolism (Clayton et al. 2009, Okuda et al. 1998, Saha et al. 1983, Meinl et al. 2009, Sousa et al. 2008). Interestingly, the composition of intestinal microbiota shows great inter-individual variability in both the presented species and the proportions of bacterial lineages (Arumugam et al. 2011a, Eckburg et al. 2005). Therefore, it is conceivable that bacteria play a role in the heterogeneity of the human response to indomethacin by modulating its metabolism and hence affecting its pharmacokinetics. However, despite a growing appreciation of the importance of intestinal microbiome, few studies have addressed its importance in indomethacin pharmacometabolomics.

The metabolism of indomethacin involves enterohepatic circulation (Harman et al. 1964). Briefly, indomethacin is glucuronidated in the liver by UDP-glucuronosyltransferases (UGTs) (Figure 4.1) and the glucuronide is delivered to the small intestine with bile acids.

In the intestine, indomethacin glucuronide is de-conjugated to the parent drug which is then reabsorbed into the circulation (Figure 4.1). Previously, bacterial β-glucuronidase expressed by intestinal microbiota has been shown to contribute to the GI damage inflicted by the anticancer drug CPT-11 (Roberts et al. 2013) and by several NSAIDs, including diclofenac, indomethacin and ketoprofen (Saitta et al. 2014).

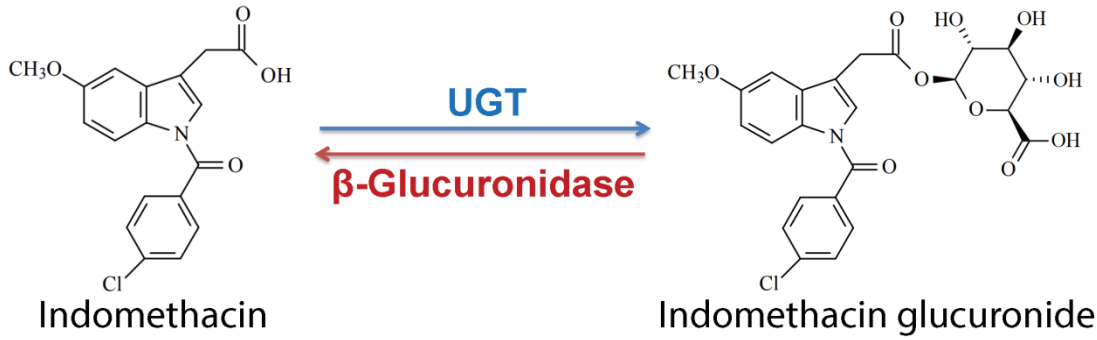

Figure 4.1 Chemical structures of indomethacin and indomethacin glucuronide. Conversion of indomethacin to indomethacin glucuronide is catalyzed by UDP-glucuronosyltransferase (UGT). The reverse de-conjugation process is catalyzed by β-glucuronidase.

Antibiotics have been widely used in research with animal models to investigate the physiological role of the normal microbiota. Using an antibiotic cocktail has provided evidence for the involvement of intestinal microbiota in various physiological and pathophysiological processes (Rakoff-Nahoum et al. 2004, Iida et al. 2013). Neomycin mainly targets Gram-negative bacteria and causes misreading of t-RNA, hence

synthesizing nonfunctional protein by binding to the bacterial 30S ribosomal subunit. Vancomycin mainly targets Gram-positive bacteria and inhibits bacterial cell-wall biosynthesis. Both neomycin and vancomycin are poorly absorbed from the gastrointestinal tract (Moellering 1984, Breen et al. 1972), and hence cause minimal interference with the host.

In this study, we use antibiotic treatment in mice as the model to address our hypotheses that the intestinal microbiota play a role in indomethacin pharmacokinetics and that this effect partially involves bacteria-mediated de-glucuronidation of indomethacin conjugate.

4.2 Materials and Methods

4.2.1 Animals

All C57BL/6 mice were purchased from the Jackson Laboratory and housed in our animal facility for at least 2 weeks before experiment. Male mice 10-14 weeks of age were used for all experiments. All animals were fed at libitum with regular chow diet (5010, LabDiet) for the course of study. Mice were kept under 12-hour light/12-hour dark (LD) cycle, with lights on at 7am and off at 7pm. Experimental protocols were reviewed and approved by the Institute for Animal Care and Use Committee at the University of Pennsylvania.

4.2.2 Study design

Mice were housed individually throughout the experiment. Mice were treated as previously described. Briefly, mice received either control (water) or an antibiotic

cocktail (1 g/L neomycin and 0.5 g/L vancomycin) for 5 days during which they have free access to a regular chow diet (Shen et al. 2015). Water for both treatment groups was spiked with aspartame. Body weight, food intake, and water intake of each mouse were followed daily throughout the study. Fecal pallets from each individual mouse were collected before, during, and after indomethacin treatment for microbiota composition analysis. 10mg/Kg indomethacin (in PEG400) was administered to mice in both antibiotic-treated and control groups at 12pm.

For the evaluation of indomethacin pharmacokinetics, blood was sampled from mouse tail veins at 1, 2, 4, 6, 8, 24, 30, 48 hours post indomethacin administration. Plasma was collected and stored at -80 ℃ for the measurement of indomethacin using liquid chromatography/mass spectrometry (LC/MS).

For the evaluation of glucuronidation, urine and fecal pellets were collected with the use of metabolic cages at 4, 8, 12, and 24 hours post indomethacin administration. Samples were stored at -80 ℃ for the measurement of indomethacin and its metabolites using LC/MS.

4.2.3 Sample preparation for mass spectrometric analysis of indomethacin

Plasma samples (~10 µl) were mixed with 50 µl indomethacin internal standard (500 ng in ACN), 20 µl formic acid and 900 µl H_2O. Samples were vortexed and centrifuged before solid phase extraction (SPE).

4.2.4 Sample preparation for mass spectrometric analysis of indomethacin metabolites

Urine samples (20 µl) were mixed with 40 µl indomethacin internal standard (40 ng in ACN), 400 µl sodium acetate and 10 µl β-Glucuronidase from Helix pomatia. After hydrolysis at 37°C for 4 h, the samples were mixed with 20 µl formic acid and 500 µl H_2O. Another urine sample (20 µl) was mixed with 40 µl indomethacin internal standard (40 ng in ACN), 20 µl formic acid and 900 µl H_2O without hydrolysis treatment. Dry stool samples were weighed before extraction with 1.7 mL of sodium acetate (0.2M, pH=5.0) using stainless steel-beads and a TissueLyser homogenizer (Qiagen, Valencia, CA, USA). The supernatant after centrifugation was divided to two aliquots. One aliquot was mixed with 40 µl indomethacin internal standard (40 ng in ACN), 20 µl formic acid and 300 µl H_2O before SPE. The other aliquot was mixed with 40 µl indomethacin internal standard (40 ng in ACN) and 15 µl β-Glucuronidase. The β-Glucuronidase hydrolysis was performed at 37 ℃ for 4 h. The samples after hydrolysis were then mixed with 20 µl of formic acid and 300 µl H_2O before SPE.

4.2.5 Solid-phase extraction

Solid-phase extraction (SPE) was performed as described in Chapter 3(3.2.6).

4.2.6 Calibration curves for mass spectrometric analysis of indomethacin

Calibration curves for indomethacin analysis were prepared as described in Chapter 3 (3.2.7).

4.2.7 Calibration curves for mass spectrometric analysis of indomethacin metabolites

To calculate the precise relative amount of indomethacin and its metabolite, standard curves were prepared in mouse urine for indomethacin, and acyl-β-D-glucuronide indomethacin (Santa Cruz Biotechnology, Dallas, Texas, USA). Individual stock solutions of each compound (100 ng/µl) were prepared in ACN and stored at -80 ℃. Working solutions were prepared by mixing equal amounts of corresponding stock solutions and performing serial dilutions with ACN. Seven point calibration samples (0, 0.032, 0.16, 0.8, 4, 20 and 100 ng/µl) for indomethacin and its metabolite were prepared. One large urine sample was obtained from mice without exposure to indomethacin. For each sample, 40 µl of (1 ng/µl) d_4-Indomethacin (Santa Cruz Biotechnology, Dallas, Texas, USA), 10 µl calibration standards were added to 20 mouse urine. The samples were extracted by SPE before LC/MS.

4.2.8 Liquid chromatography/mass spectrometry for indomethacin

Indomethacin was measured by LC/MS as described in Chapter 3 (3.2.8).

4.2.9 Liquid chromatography/mass spectrometry for indomethacin metabolites

Indomethacin metabolites were measured using a TSQ Quantum Ultra™ triple quadrupole mass spectrometer (Thermo Scientific, Wilmington, DE, USA) equipped with an ESI ion source. The Mass Spectrometer was connected to a Thermo Scientific Accela HPLC Systems and equipped with a PAL auto sampler and thermocontroller (set at 4 ℃).

The CSH C18 Column (2.1 mm X 150 mm, 130Å, 1.7 μm, Waters) was used at a constant 40 ℃. The mobile phase (A) (90% H_2O/10% (B), 0.2% acetic acid) and mobile (B) (90% ACN/10% methanol) was used at a flow rate of 350 μl/min with a binary gradient (0-12 min, 10-50% B; 12-12.5 min, 50-100% B; 12.5-16 min, 100% B; 16.2-20 min, 10% B). Mass spectrometry was performed in negative mode. The transition for indomethacin and d_4-indomethacin are 355.9>311.9 and 359.9>315.9, respectively. The transition for O-desmethyl indomethacin and acyl-β-D-glucuronide indomethacin are 298.0>256.1 and 533.1>193.3, respectively. Both Q1 and Q3 were operated at 0.7 m/z FWHM. Peak area ratios of target analytes to d_4-indomethacin internal standards were calculated by Xcalibur Quan software. The data were fitted to the calibration curves to calculate the precise relative amount of indomethacin and its metabolites.

4.2.10 DNA extraction for microbiota composition analysis

DNA extraction was performed as described in Chapter 2 (2.2.3).

4.2.11 16S rRNA quantification

Quantification of 16S rRNA was performed by real-time PCR as described in Chapter 2 (2.2.4).

4.2.12 V1-V2 16S rRNA region amplification and sequencing

16S rRNA gene amplification was performed as described in Chapter 2 (2.2.5).

4.2.13 Bioinformatics

Sequence data were processed with QIIME v 1.8.0 (Caporaso et al. 2010c) using default parameters, as described in Chapter 2 (2.2.6).

4.2.14 Pharmacokinetic analysis

Plasma indomethacin concentrations at 1, 2, 4, 6, 8, 24, 30, 48 hours post administration were plotted against time to generate the "plasma indomethacin concentration versus time curve". The area under the curve (AUC_{total}) was then calculated according to the trapezoidal rule and the elimination rate constant (K_{el}) was obtained as the slope value. The half-life ($t_{1/2}$) was calculated as $t_{1/2} = ln2/k_{el}$. The apparent volume of distribution V_d was calculated as $V_d = dose/C_0$. C_0 was extrapolated using the plasma drug concentration versus time curve. The oral clearance Cl was calculated as $Cl = dose/AUC_{total}$.

4.3 Results

4.3.1 Antibiotic-treatment depletes the microbiota in the intestine

The 16S rRNA copy numbers mice receiving control water was relatively stable throughout the study. However, the 16S rRNA copy numbers in feces were reduced by 99.9% in mice receiving antibiotic cocktail after 5 days (Figure 4.2 A). After cessation of antibiotic cocktail, the 16S rRNA copy number increased slightly, but was still reduced by 93.8% on Day 6 and 87.5% on Day 7 (Figure 4.2 A).

Microbial diversity analysis revealed a significant decrease, starting at Day 4. The observed species was reduced by 86.7% on Day 4, 89.6% on Day 5, 91.8% on Day 6, and 97.2% on Day 7 (Figure 4.2 B).

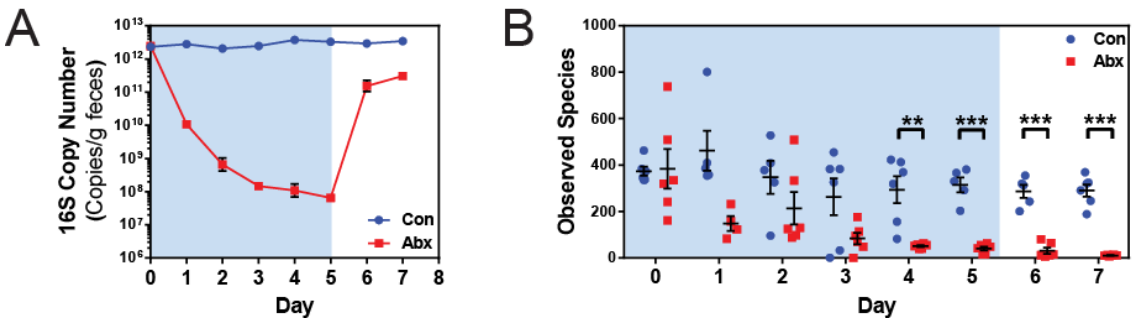

Figure 4.2 Antibiotic-treatment reduces the gut microbial load in mice. Mice were treated with the antibiotic cocktail (Abx, neomycin and vancomycin) or control water (Con) for 5 days (blue-shaded area) and their fecal microbiota compositions over time were analyzed using 16S rRNA profiling. **(A)** Longitudinal analysis of 16S rRNA gene copies per gram of feces reveals a significant reduction in microbial load in Abx group (red). **(B)** Longitudinal analysis of Observed Species reveals decreased microbial richness in Abx group (red). ** $p < 0.01$, *** $p < 0.001$ by multiple t test, FDR corrected. N=4-6/group. Mean ± S.E.M. shown.

During antibiotic treatment, body weight, food intake, and water intake were measured daily. As shown in Figure 4.3, antibiotic-treated mice had comparable body weight, food intake and water intake over the time-course studied with control mice. Higher water

intake on Day 5 in antibiotic-treated mice was attributed to water bottle movement when the facility staff changed the cages.

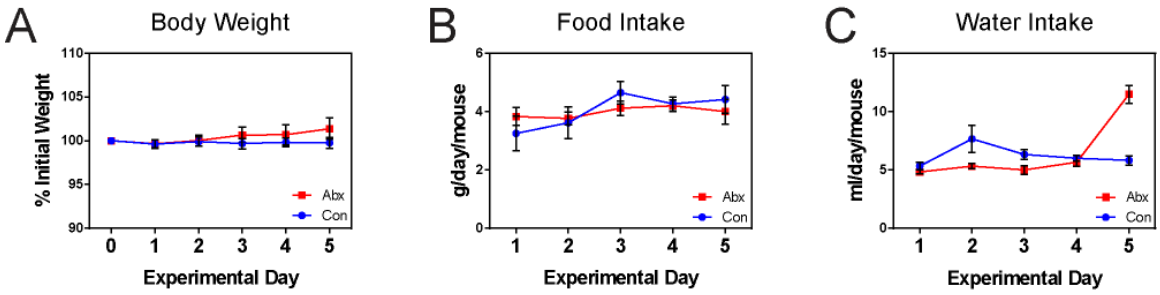

Figure 4.3 Body weight, food intake, and water intake are not affected by antibiotic-treatment in C57BL/6 mice. (A) Body weight, **(B)** Food intake, and **(C)** Water intake are not affected by antibiotic treatment. Note: on day 5 animal cages were changes by facility staff and the movement caused water loss. N=6/group. Mean ± S.E.M. shown.

4.3.2 Microbiota composition is altered by antibiotic-treatment

Besides comparable microbial biomass and diversity prior to antibiotic treatment, all mice had similar composition of fecal microbiota as well (Figure 4.4, Day 0). After 5 days of antibiotic treatment, mice showed significantly shifted the composition of the fecal microbiota, with a reduction in Bacteroidetes and Firmicutes, and a concomitant expansion of Proteobacteria (Figure 4.4).

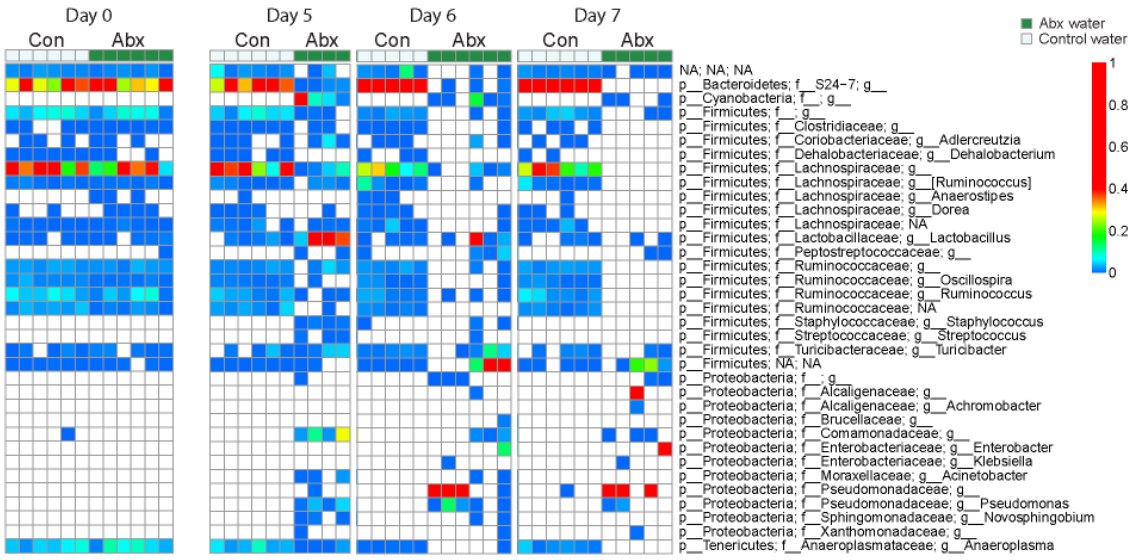

Figure 4.4 Antibiotic treatment alteres the microbiota composition. Heat map of the longitudinal analysis of bacterial lineages detected in feces of control (light green) or antibiotic treated (dark green) mice before (Day 0) and after (Day 5, 6, and 7) treatment. Each column represents one individual mouse of the time and treatment group indicated. Microbial composition is shifted in the Abx group but is stable in Con group. The proportions of bacterial lineages are indicated by the color code to the right.

4.3.3 Indomethacin pharmacokinetics are altered in antibiotic-treated mice

In antibiotic-treated mice, the oral clearance of indomethacin was increased by 19.6% (Figure 4.5 A), and the elimination rate constant K_{el} was increased by 55.2% (Figure 4.5 B), indicating an increased elimination of indomethacin. Consistently, the total area-under-the-curve (AUC_{total}) of indomethacin, which is a measurement of total drug

exposure, was decreased by 16.8% in antibiotic-treated mice (Figure 4.5 C). The half-life ($t_{1/2}$) of indomethacin was decreased by 37.5% (Figure 4.5 D), and the apparent volume of distribution (V_d) of indomethacin was decreased by 46.1% (Figure 4.5 E) in antibiotic-treated mice.

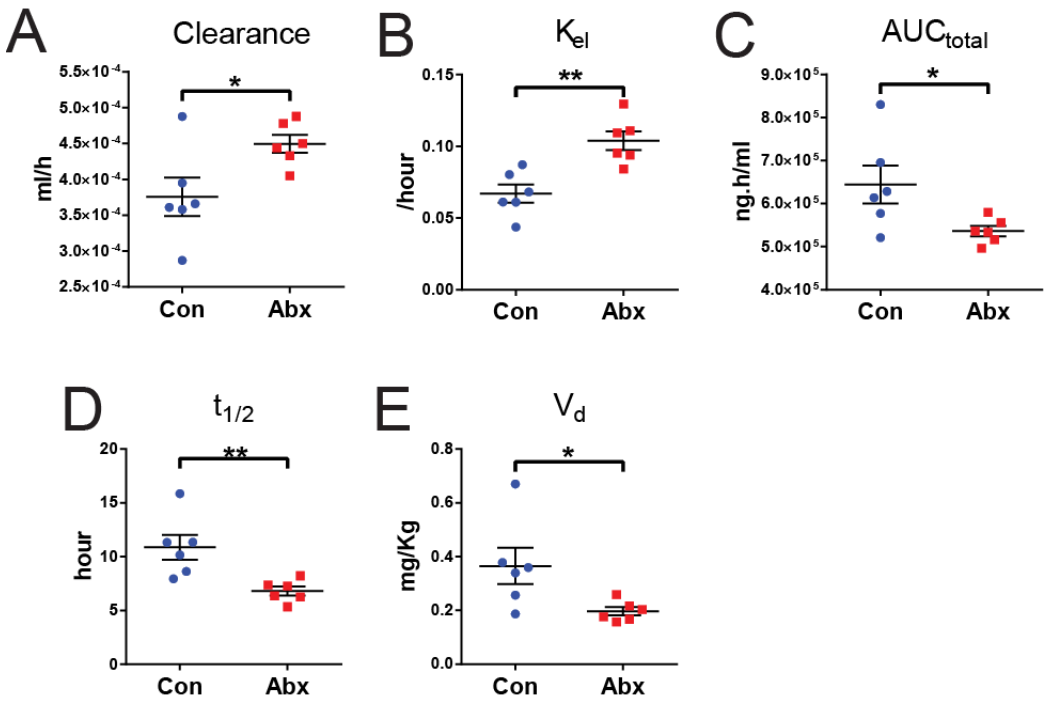

Figure 4.5 The pharmacokinetics of indomethacin is altered in antibiotic-treated mice. In antibiotic-treated mice (red), indomethacin has increased **(A)** oral Clearance and **(B)** elimination rate constant (K_{el}), as well as decreased **(C)** area under the curve (AUC_{total}), **(D)** half-life ($t_{1/2}$), and **(E)** apparent volume of distribution (V_d). * $p < 0.05$, ** $p < 0.01$ by Mann-Whitney test. N=6/group. Mean ± S.E.M. shown.

The variances of the total area-under-the-curve (AUC_{total}), half-life ($t_{1/2}$), and the apparent volume of distribution (V_d) of indomethacin were significantly smaller in antibiotic-treated mice than in control mice (p= 0.01 for AUC_{total}, p=0.04 for $t_{1/2}$, and p=0.006 for V_d by F test), suggesting antibiotic-treatment reduced the source of variation in response to indomethacin.

4.3.4 Indomethacin glucuronidation in antibiotic-treated mice is impaired

Detection of indomethacin and indomethacin-glucuronide was confirmed by incubating samples with or without β-glucuronidase.

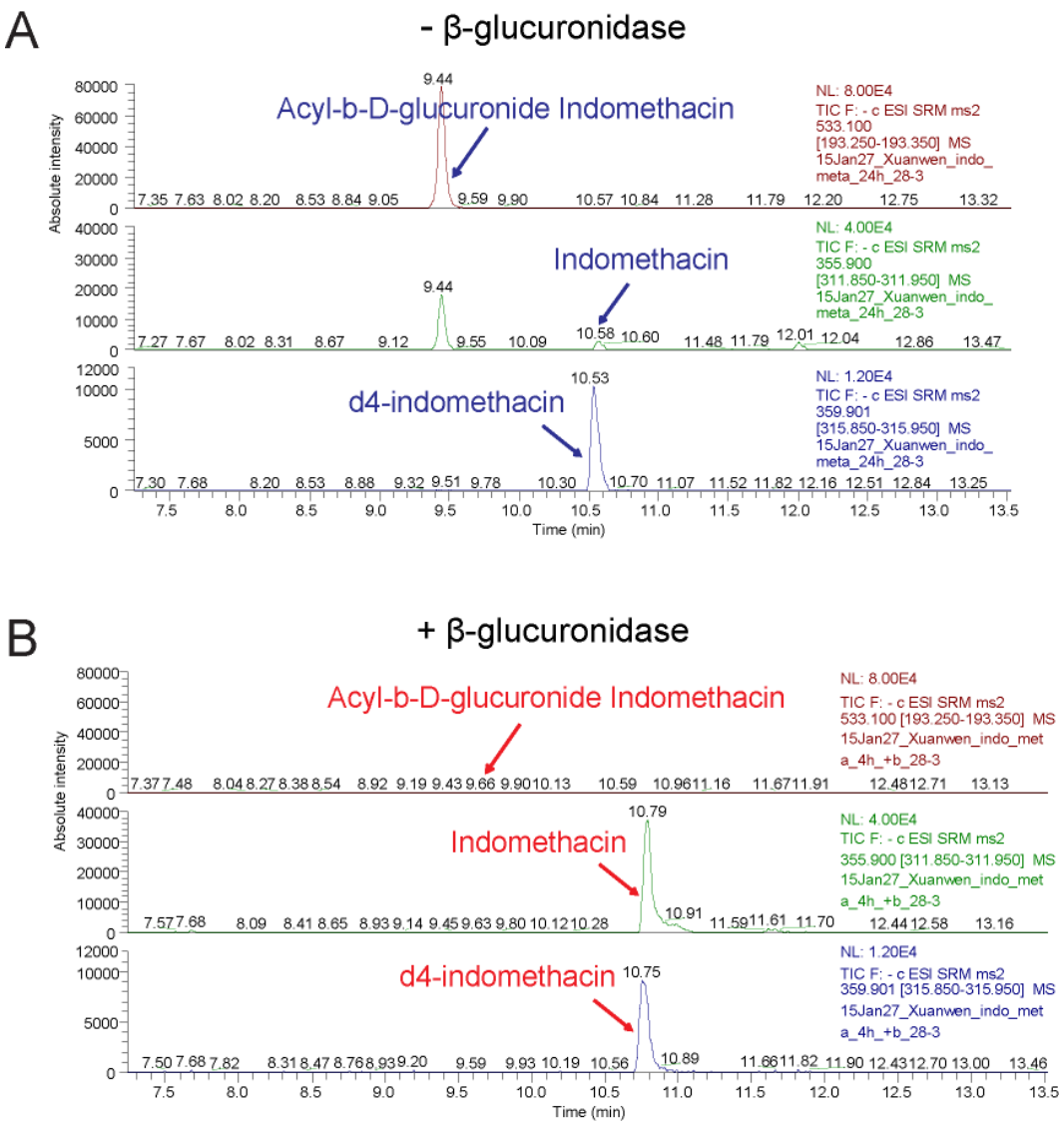

Figure 4.6 Representative spectra of LC/MS measurements of indomethacin and its metabolites. (A) Samples without β-glucuronidase incubation. The peak denoting Acyl-b-D-glucuronide Indomethacin (indomethacin glucuronide) was larger, whereas the peak denoting Indomethacin was smaller. **(B)** Samples with β-glucuronidase incubation. The peak denoting Acyl-b-D-glucuronide Indomethacin was diminished, whereas the peak denoting Indomethacin was increased.d_4-indomethacin is the internal standard for indomethacin.

As shown in the representative spectra (Figure 4.6), there was a large peak corresponding to indomethacin glucuronide and a smaller peak corresponding to indomethacin in the control samples, whereas after incubation with β-glucuronidase, the peak of indomethacin glucuronide was diminished and that of indomethacin greatly increased. This verified our mass spectrometry detection of indomethacin and its metabolites.

This shift of decreased indomethacin concentration and increased indomethacin glucuronide concentration was detected in each of the samples we studied as shown in Figure 4.7. Indomethacin glucuronide was readily detected in the absence of incubation with β-glucuronidase. However, after incubation with β-glucuronidase, only indomethacin was detected in samples, indicating the breakdown of indomethacin glucuronide by β-glucuronidase.

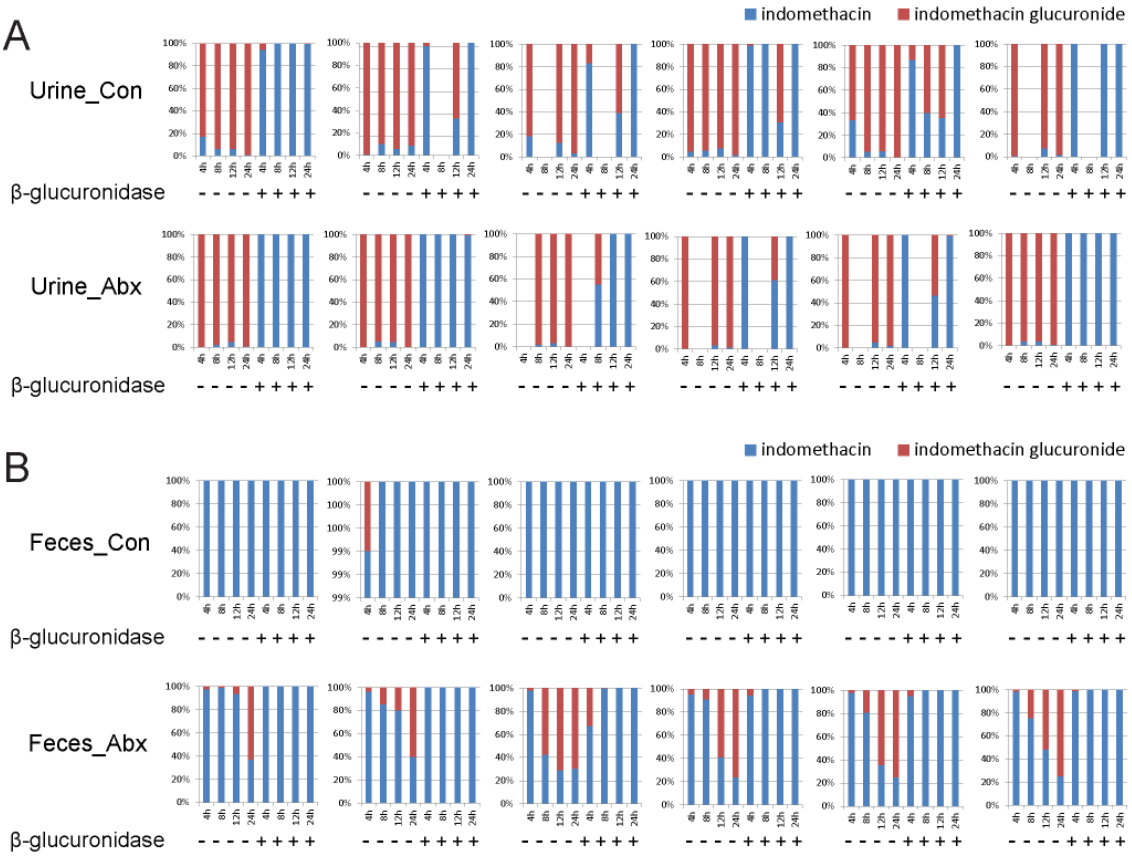

Figure 4.7 Proportions of indomethacin and indomethacin glucuronide in samples with or without β-glucuronidase incubation. (A) Urine samples of both control mice (Con) and antibiotic-treated mice (Abx). **(B)** Fecal samples of both control mice (Con) and antibiotic-treated mice (Abx). The proportions of indomethacin glucuronide (red) are smaller with β-glucuronidase added. Similarly, the proportions of indomethacin (blue) are larger with β-glucuronidase added. Each graph shows the longitudinal changes in one mouse. N=6/group.

Figure 4.7 also showed that antibiotic-treated mice had more indomethacin glucuronide in both urine and feces compared to control mice, indicating the glucuronidation level of indomethacin was altered due to the depletion of intestinal microbiota. To address this question, we compared the ratio of indomethacin-glucuronide to indomethacin in mice pretreated with or without the antibiotic cocktail.

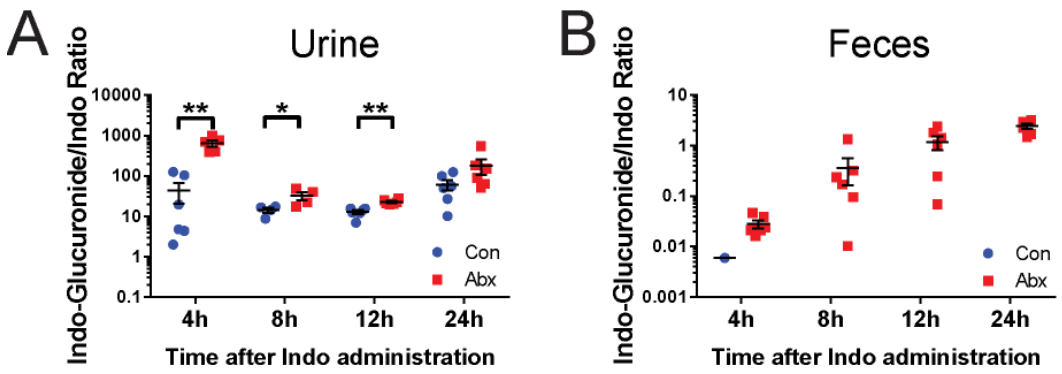

Figure 4.8 Glucuronidation level of indomethacin in antibiotic-treated mice are altered. (A) The ratio of indomethacin-glucuronide to indomethacin in urine of control mice (blue) and antibiotic-treated mice (red) following indomethacin administration. **(B)** The ratio of indomethacin-glucuronide to indomethacin in urine of control mice (blue) and antibiotic-treated mice (red) following indomethacin administration. In both urine and feces, the ratio is higher in antibiotic-treated mice. * $p < 0.05$, ** $p < 0.01$ by Mann-Whitney test. N=6/group. Mean ± S.E.M. shown.

In urine, the ratio was significantly higher in antibiotic-treated mice for the first 12 hours following indomethacin administration (Figure 4.8 A). The ratio in antibiotic-treated

mice is 15 fold at 4h, 2.5 fold at 8h, and 1.7 fold at 12h of that in control mice. In feces, indomethacin-glucuronide was barely detectable in control mice, yet was steadily detected in antibiotic-treated mice (Figure 4.8 B).

Indomethacin suppressed urinary prostanoid metabolites irrespective of treatment with the antibiotic cocktail (Figure 4.9). PGD-M and PGI-M levels were reduced by indomethacin in a time-dependent fashion in both control mice and antibiotic-treated mice. However, ANOVA revealed a significant effect due to antibiotic treatment in the more abundant urinary PGE-M and Tx-M concentrations. Unlike in control mice, where both metabolites were reduced time-dependently, in antibiotic-treated mice, both metabolites were detectably suppressed to a lesser degree by indomethacin and their concentration started to recover faster in antibiotic-treated mice compared to control mice (Figure 4.9). The reduced inhibitory effect was attributable to the reduced distribution and exposure to indomethacin in antibiotic-treated mice. Taken together, the β-glucuronidase-catalyzed de-glucuronidation was impaired due to antibiotic-treatment, partially suppressing the inhibitory effect of indomethacin on COX enzymes.

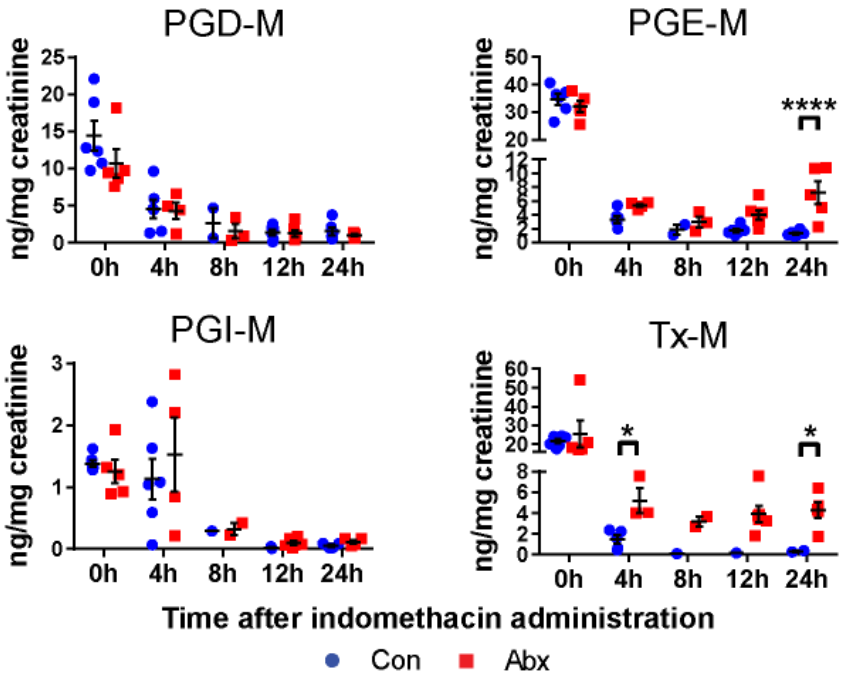

Figure 4.9 Efficacy of indomethacin in antibiotic-treated mice is altered. Urinary prostanoid metabolites were analyzed with LC/MS and values are corrected by creatinine. In control mice (blue), all metabolites were reduced time-dependently. In Abx mice (red) PGD-M and PGI-M remained suppressed 24 hour after indomethacin, whereas PGE-M and Tx-M concentrations recovered more quickly. Two-way ANOVA revealed significant effect of time in PGD-M (p=0.001) and PGI-M (p=0.0004), and significant antibiotic effect of PGE-M (p<0.0001) and Tx-M (p=0.0002).In abx mice, PGE-M was higher mice at 24h, and Tx-M was higher at 4h and 24h. N=6/group. * $p < 0.05$, ** $p < 0.01$ by multiple comparison test, adjusted. Mean ± S.E.M. shown.

4.4 Discussion

Indomethacin undergoes enterohepatic circulation (Harman et al. 1964). It is glucuronidated in the liver by UDP-glucuronosyltransferases (UGTs) and the glucuronide is delivered to the small intestine with bile acid. The intestinal bacteria have been shown to have functional β-glucuronidase (Roberts et al. 2013). Here we show that when the intestinal microbiota was suppressed by an antibiotic cocktail, the level of de-glucuronidation was significantly compromised (Figure 4.8), consistent with involvement of intestinal bacteria in the catalysis of this reaction. Therefore, the intestinal microbiota, by playing a role in the metabolism of indomethacin, have an impact on indomethacin pharmacokinetics. When bacteria-mediated de-glucuronidation was impaired, indomethacin reabsorption into the circulation was reduced, consistent with a shortened half-life and reduced drug exposure (Figure 4.5). Suppression by indomethacin of the urinary excretion of the most abundant prostanoid metabolites - Tx and PGE - was detectably reduced in antibiotic treated mice, suggesting that concomitant antibiotic treatment may undermine the efficacy of this NSAID. It may also explain the attenuated enteropathy associated with indomethacin in rats pretreated with antibiotics (Koga et al. 1999a).

The association of the genetic variation of Cytochrome P450 2C9 (CYP2C9) with the GI complications of indomethacin is unaddressed, although indomethacin clearance is largely catalyzed by CYP2C9 (Nakajima et al. 1998). A small study reported that there is no clinically significant relationship between CYP2C9 genotype and risk of gastric ulceration resulting from NSAID usage (Martin et al. 2001). However, its conclusion is

limited by the small sample size and the miscellaneous NSAIDs. Due to the lack of evidence, pharmacogenetic testing for indomethacin was not clinically recommended (Wyatt, Pettit & Harirforoosh 2012).

Since the intestinal microbiota are variable amongst individuals (Arumugam et al. 2011b, Bushman, Lewis & Wu 2013), intestinal microbiota-mediated metabolism of indomethacin may be a potential factor underlying the inter-individual differences of indomethacin pharmacokinetics (Brune 1985, Brune 1987). Indeed, we found that antibiotic-treated mice have significantly less inter-mouse variability of half-life and volume of distribution compared to control mice (Figure 4.5 D, E). Despite this work still being at an early in the stage, consideration should be given to intestinal microbiota-mediated biotransformation of drugs during drug development as at least 30 drugs are directly or indirectly affected by intestinal bacteria (Clayton et al. 2009, Okuda et al. 1998, Saha et al. 1983, Meinl et al. 2009, Sousa et al. 2008).

Chapter 5 Rhythmicity of the intestinal microbiota is regulated by gender and the host circadian clock

5.1 Introduction

Despite influences from host genetics (Benson et al. 2010b), ageing (Biagi et al. 2010), use of antibiotics (Jernberg et al. 2010), lifestyle (Annalisa et al. 2014), pet ownership (Song et al. 2013), and concomitant disease (Zhao 2013, Wu, Bushman & Lewis 2013), the intestinal microbial community is largely shaped by diet (Wu et al. 2011) and the impact has been well established in both humans and mice (David et al. 2014, De Filippo et al. 2010).

In addition to the type of food consumed (Daniel et al. 2014), the feeding behavior of the host also influences the microbiota. For instance, a 24-hour fast increases the abundance of Bacteroidetes and reduces that of Firmicutes in mouse cecum, without altering the communal microbial diversity (Crawford et al. 2009). Bacteroidetes are also dominant in the microbiota of the fasted Burmese python, while ingestion of a meal shifts the intestinal composition towards Firmicutes (Costello et al. 2010).

It has been widely appreciated that the pharmacokinetics of orally dosed indomethacin shows circadian variation both in humans and in rats. For instance, a reduced peak in the plasma concentrations of indomethacin associated with a prolonged apparent half-life ($t_{1/2}$) and with an increased formation of indomethacin metabolites was observed in patients dosed in the evening compared to those dosed in the morning or at noon (Guissou et al. 1983). Interestingly, a double-blind clinical study showed that indomethacin caused less

undesirable effects in patients dosed in the evenings compared to those dosed in the morning (Levi, Le Louarn & Reinberg 1985). Moreover, rats dosed during the rest phase have significantly lower initial indomethacin plasma concentrations (C_0) and area under the curve (AUC), as well as significantly increased distribution volume (V_d) and total metabolic clearance (Cl_T) compared to rats dosed during the active phase (Guissou et al. 1987).

We observed that the intestinal microbiota affects the pharmacokinetics and pharmacodynamics of indomethacin in mice partially via bacteria-mediated de-glucuronidation (Chapter 4). A recent study used a functional metagenomic approach and revealed a class of β-glucuronidases that may be part of a functional core with major health implications (Gloux et al. 2011). Therefore, it is worthwhile to investigate whether the effect of intestinal microbiota on indomethacin pharmacokinetics and efficacy is a potential factor contributing to the chronopharmacology of indomethacin via diurnal oscillation in its composition.

In this study, we address our hypotheses that the microbial composition oscillates diurnally and this oscillation is influenced by the host clock.

5.2 Materials and Methods

5.2.1 Animals

All C57BL/6 mice and *Bmal1* KO mice were raised in our animal facility. Mice 10-14 weeks of age were used for all experiments. All animals were fed *at libitum* with regular chow diet (5010, LabDiet) for the course of study. Mice were kept under 12-hour

light/12-hour dark (LD) cycle, with lights on at 7am and off at 7pm. Experimental protocols were reviewed and approved by the Institute for Animal Care and Use Committee at the University of Pennsylvania.

5.2.2 Study design

Study 1: Microbial oscillation in WT mice. C57BL/6 mice were bred in our facility and caged according to litter and gender. 7 male mice (housed in two cages with 3 in one cage and 4 in the other) and 7 female mice (housed in two cages with 3 in one cage and 4 in the other) were studied. Fecal pellets from each mouse were sampled every 4 hours for 48 hours (2 light-dark cycles, 13 time points), as illustrated in Figure 5.1. A total of 182 samples were collected for microbiota composition analysis by pyrosequencing. Sequencing yielded 360,809 reads.

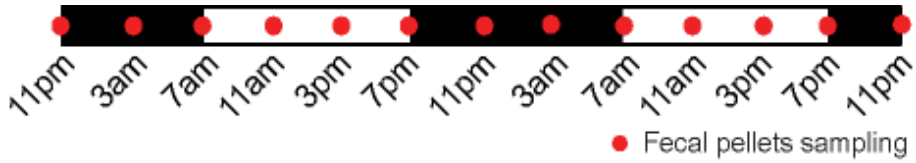

Figure 5.1 Schematic diagram illustrating the study design. Fecal pellets were longitudinally sampled from individual mice at 11pm, 3am, 7am, 11am, 3pm, and 7pm for 2 continuous light-dark cycles as indicated by the red dot. All fecal pellets were weighed and stored at -80 ℃ until use.

Study 2: Effect of Bmal1 deletion on microbial oscillation in mice. Homozygous *Bma1* KO mice were established in our mouse colony by mating heterozygous pairs. *Bmal1* KO mice were housed separately from the littermate wild type (WT) controls as soon as the genotype was confirmed to minimize the influence of corporophagia (Deloris Alexander et al. 2006). Four female (housed in two cages with 2 in each) and two male (housed in two cages with 1 in each) *Bmal1* KO mice as well as two female (housed in two cages with 1 in each) and three male (housed in two cages with 2 in one cage and 1 in the other) littermate control mice were studied. Fecal pellets from each mouse were sampled every 4 hours for 48 hours, as illustrated in Figure 5.1. A total of 143 samples were collected for microbiota composition analysis by pyrosequencing.

5.2.3 DNA extraction for microbiota composition analysis

DNA extraction was performed as described in Chapter 2 (2.2.3).

5.2.4 16S rRNA quantification

Quantification of 16S rRNA was performed by real-time PCR as described in Chapter 2 (2.2.4).

5.2.5 V1-V2 16S rRNA region amplification and sequencing

16S rRNA gene amplification was performed as described in Chapter 2 (2.2.5).

5.2.6 Bioinformatics

Sequence data were processed with QIIME v 1.8.0 (Caporaso et al. 2010c) using default parameters, as described in Chapter 2 (2.2.6).

5.2.7 Statistical analysis

Statistical tests for rhythmicity were performed using JTK_CYCLE algorithm as previously described (Hughes, Hogenesch & Kornacker 2010) with a period-length window of 20–28 hr. Comparisons of the relative abundances of bacterial taxons were done by Mann-Whitney test. Paired tests were performed where appropriate. *p*-values were corrected for multiple comparisons using the procedure of Benjamini and Hochberg to control for a false discovery rate (FDR) of less than 5%. PERMANOVA (McArdle, Anderson 2001) procedure was applied based on pairwise UniFrac distance to assess the sources of variation, and p-values were obtained using permutation tests.

5.3 Results

5.3.1 Microbiota composition oscillates diurnally

The relative abundances of Bacteroidetes and Firmicutes, the two most abundant components of mammalian microbiota, varied during the light-dark cycle (Figure 5.2). The average abundance of Bacteroidetes was higher at 11pm (66%) and 11am (60%) and lower at other times, whereas that of Firmicutes was higher at 3am (45%) and 7am (45%) and lowest at 11pm (29%).

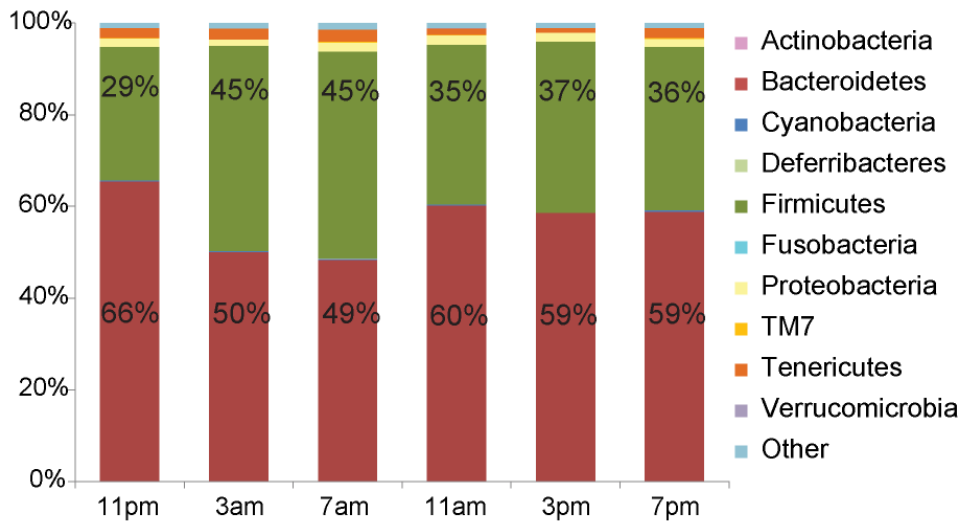

Figure 5.2 Bar graphs of average relative abundances of bacterial phyla of WT mice. Fecal pellets from male and female mice housed under normal light-dark cycle were collected every 4 hours for 48 hours. Microbiota structure in fecal samples of combined genders at various sampling time points during the day were plotted. Lineages were identified by the uclust consensus taxonomy classifier.

We used the JTK_CYCLE algorithm (Hughes, Hogenesch & Kornacker 2010) to test whether the composition of fecal microbiota exhibited circadian rhythmicity. The fecal bacterial load, as measured by the total 16S rRNA gene copy numbers in samples, oscillated diurnally during the light-dark cycle both in the group as a whole and when segregated by gender (Figure 5.3 A). The bacterial load peaked around 11pm and gradually decreased towards the late dark phase until the lowest level was noted around 7am when the light was on. Male mice had more bacteria overall in feces than female

mice, however female mice showed more significant diurnal oscillation (p=2.82E-06) than male mice (p= 0.03).

Diurnal oscillation was also observed in the relative abundance of Bacteroidetes and Firmicutes (Figure 5.3 B, C). Bacteroidetes were most abundant at 11pm and least abundant at 7am. Firmicutes exhibited a contrasting pattern and accordingly, the ratio of Bacteroidetes to Firmicutes exhibited diurnal oscillation (Figure 5.3 D). The average gastrointestinal transit time of mice is about 4-5 hours (Kashyap et al. 2013). Hence the fecal pellets we sampled are representative of the microbiota of the host 4-5 hours before sampling time. As a result, the peak and trough of the relative abundance of Baceroidetes in the upper gastrointestinal tract should be around 7pm and 3am, respectively. Similarly, the peak and trough of the relative abundance of Firmicutes in the upper gastrointestinal tract should be around 3am and 7pm, respectively. The inferred absolute abundance of bacterial lineage was calculated by multiplying its relative abundance by the 16S rRNA gene copy number in each sample.

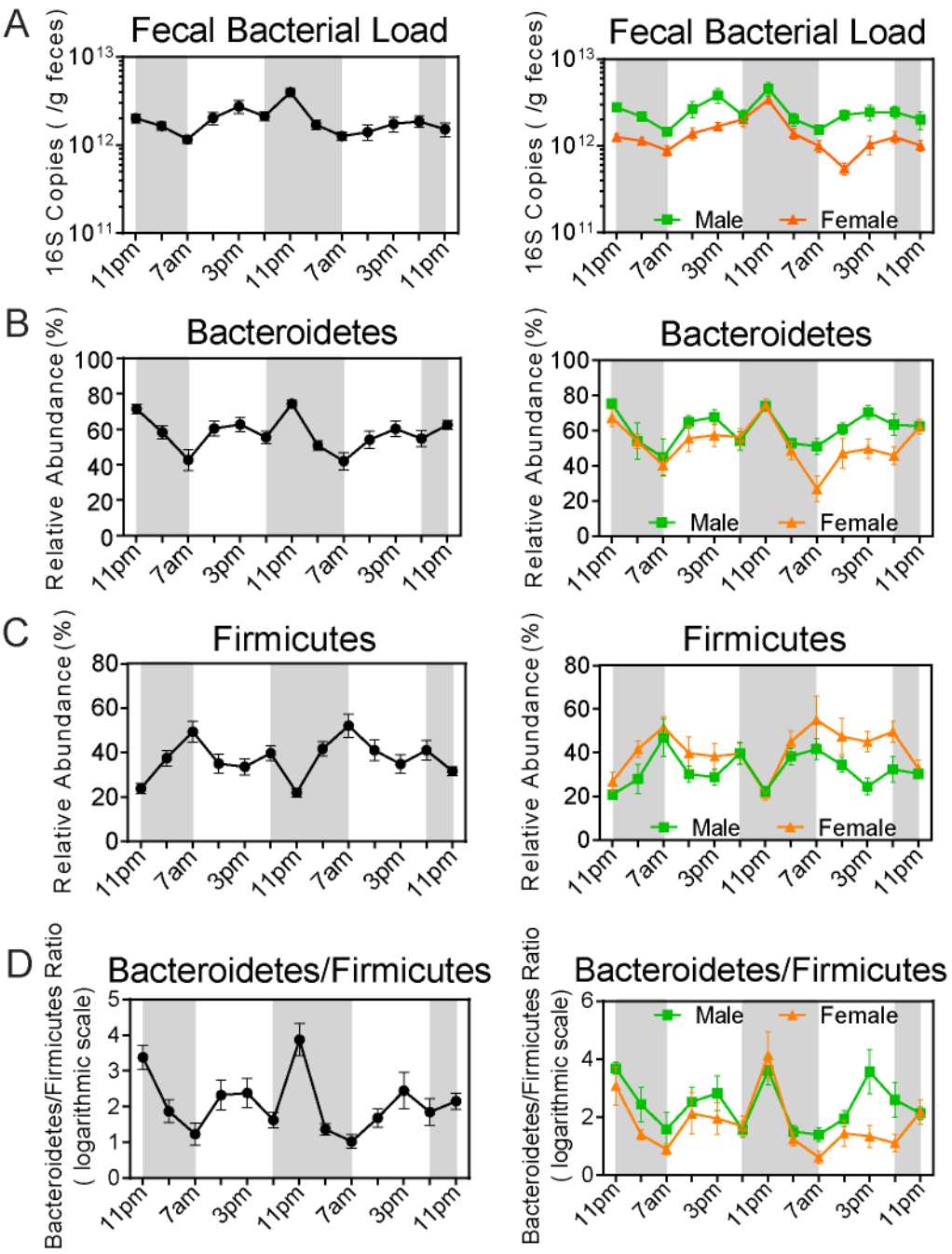
A
Fecal Bacterial Load
16S Copies (/g feces)
10^{13}
10^{12}
10^{11}
11pm
7am
3pm
11pm
7am
3pm
11pm
Male
Female
B
Bacteroidetes
Relative Abundance (%)
100
80
60
40
20
0
C
Firmicutes
D
Bacteroidetes/Firmicutes
Bacteroidetes/Firmicutes Ratio
(logarithmic scale)
5
4
3
2
1
0
6

Figure 5.3 Diurnal oscillation of intestinal microbiota composition in C57BL/6 mice. The light phase is indicated as white, the dark phase indicated by grey shading. **(A)** Fecal bacterial load oscillates diurnally in mice both when combined (left) and separated (right; male, green; female, orange) by gender, as indicated by 16S rRNA copy numbers normalized to sample weight. JTK_ CYCLE revealed p=0.0001 for mice of combined genders, p= 0.03 for male mice, and p=2.82E-06 for female mice. **(B)** Relative abundance of Bacteroidetes oscillates diurnally in mice when genders are combined (left) and separated (right). JTK_ CYCLE revealed p=0.0001 for combined genders, p= 0.03 for male mice, and p=0.0006 for female mice. **(C)** The relative abundance of Firmicutes oscillates in an opposite pattern to Bacteroidetes in mice of combined (left) and separated (right) genders. JTK_ CYCLE revealed p=6.29E-05 for mice when genders are combined, p= 0.05 for male mice, and p=0.001 for female mice. **(D)** The ratio of Bacteroidetes to Firmicutes oscillates accordingly in mice of combined (left) and separated (right) genders. JTK_CYCLE revealed p=5.84E-05 for mice of combined genders, p= 0.04 for male mice, and p=0.0009 for female mice. $N_{male} = 7$, $N_{female} = 7$. Mean ± S.E.M. shown.

Examination of the relative abundances at the genus level revealed more structure in the taxonomic oscillation (Figure 5.4). *S24-7 spp.*, a major genus of Bacteroidetes, oscillates in the same pattern as Bacteroidetes (Figure 5.3 B). Several abundant genera in Firmicutes, including *Lachnospiraceae spp., Oscillospira, Ruminococcaceae spp., Clostridiales spp.*, and *Clostridia spp.*, oscillate out of phase with *S24-7 spp.* *Anaeroplasma*, a Tenericutes, oscillates in phase with Firmicutes. Thus, the composition

of microbiota in C57BL/6 mice, both total bacterial load and the abundance of discrete bacterial lineages, undergoes diurnal oscillation during the 24-hour light-dark cycle.

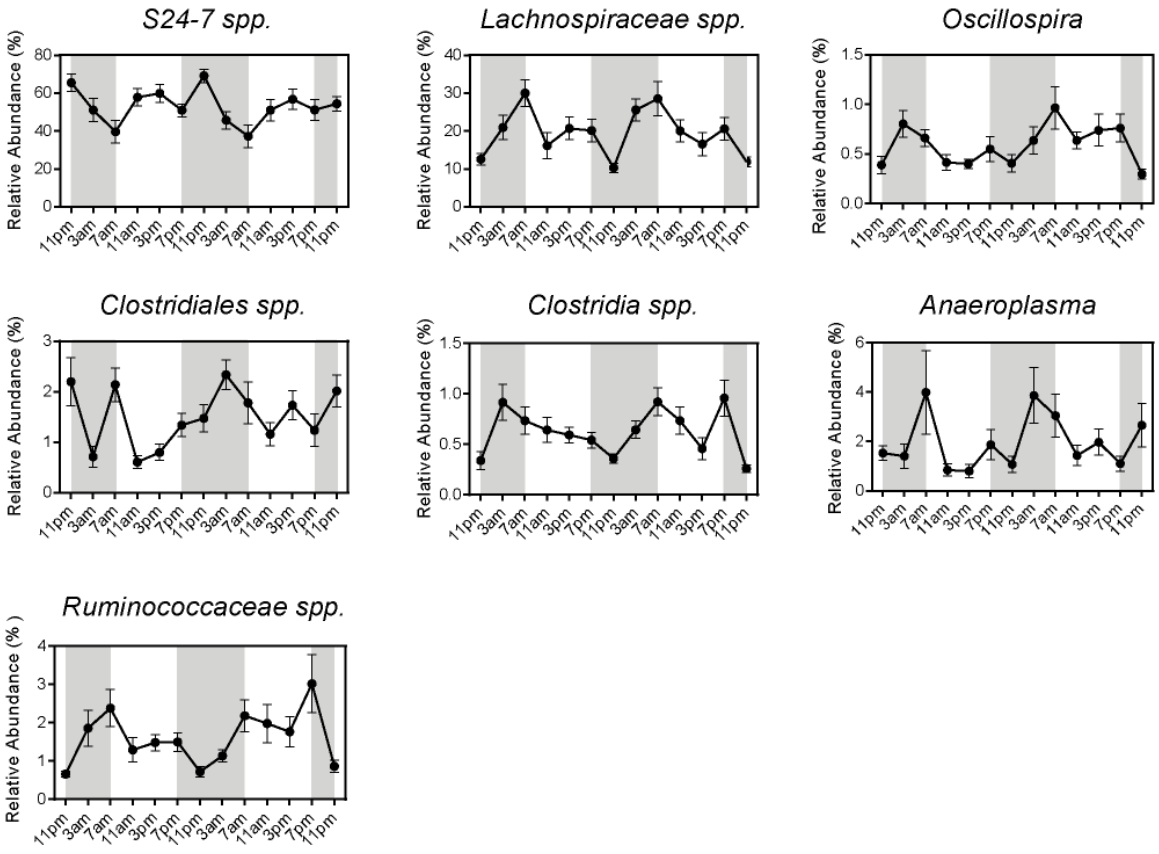

Figure 5.4 Diurnal oscillation of genus abundances in C57BL/6 mice. Diurnal oscillations during the light-dark cycle were observed in the abundances of *S24-7 spp.* (p=0.016), *Lachnospiraceae spp.* (p=0.0004), *Oscillospira* (p=0.0005), *Clostridiales spp.* (p=0.002), *Clostridia spp.* (p=0.001), and *Anaeroplasma* (p=0.03), and *Ruminococcaceae spp.* (p=0.003).

5.3.2 *Bmal1* deletion abolishes the oscillation in microbiota composition

Fecal pellets were sampled from *Bmal1* knock out (KO) mice and their littermate control mice every 4 hours for 48 hours, to investigate the effect of *Bmal1* deletion.

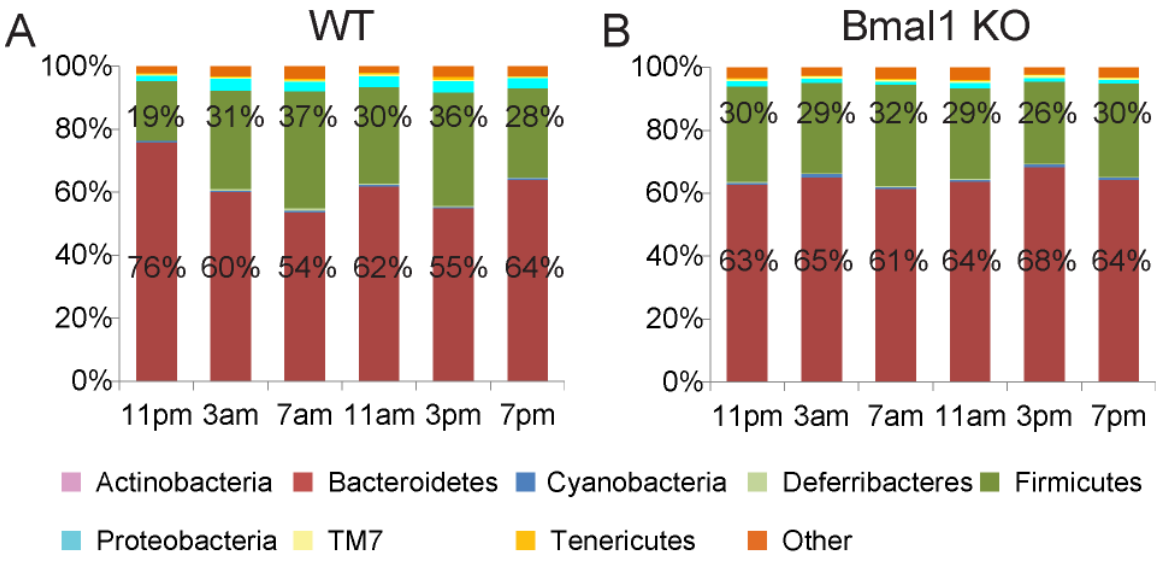

Figure 5.5 Bar graphs of average relative abundances of bacterial phyla of WT and *Bmal1* KO mice. (A) Microbiota structure in fecal samples of combined genders of WT mice at various sampling time points during the day were plotted. **(B)** Microbiota structure in fecal samples of combined genders of *Bmal1* KO mice at various sampling time points during the day were plotted.

Circadian rhythms in the microbiota differed in *Bmal1* KO mice versus wild type (WT) mice (Figure 5.5). In WT mice, the average abundance of Bacteroidetes was highest at 11pm (76%) lowest at 7am (54%), and that of Firmicutes was highest at 7am (37%) and

lowest at 11pm (19%). In contrast, variability in the composition of the microbiota was suppressed in *Bmal1* KO mice (Figure 5.5 B).

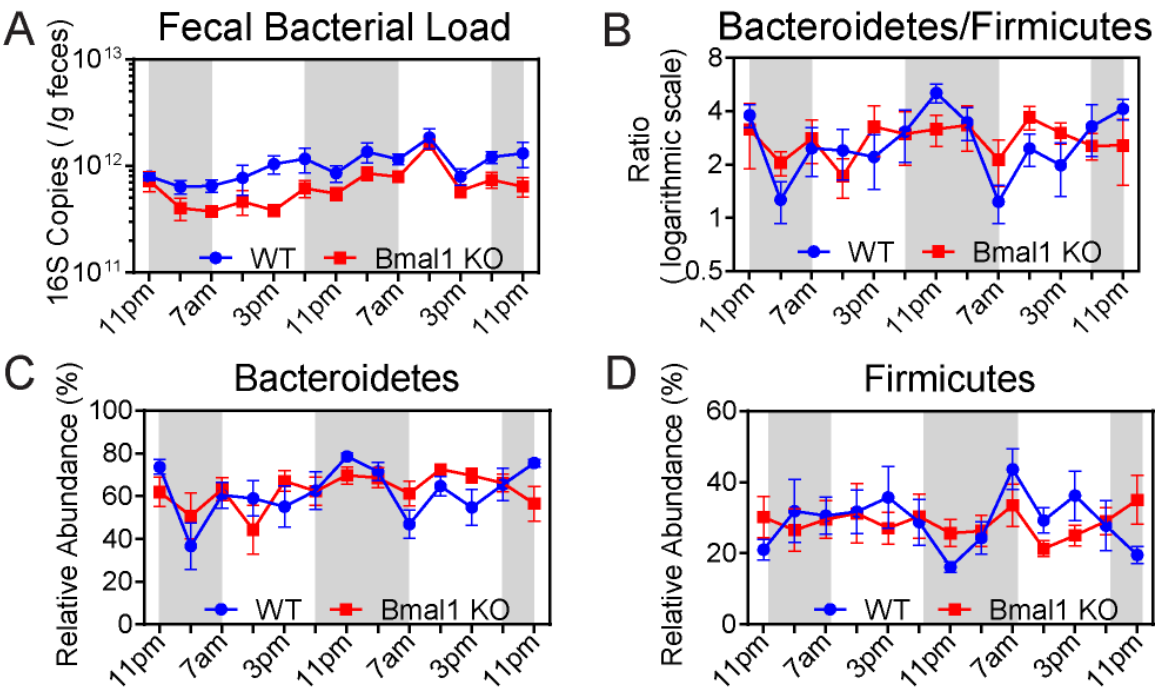

Figure 5.6 *Bmal1* deletion abolishes the diurnal oscillation of intestinal microbiota composition in mice. (A) The fecal bacterial load in *Bmal1* KO mice (red) was slightly lower than that in WT mice (blue) but showed a similar fluctuating pattern during the light-dark cycle. **(B)** The ratio of Bacteroidetes to Firmicutes oscillates diurnally in WT mice (blue; p=0.01) but not in Bmal1 KO mice (red). **(C, D)** The relative abundances of Bacteroidetes and Firmicutes oscillate diurnally in WT mice (blue; p=0.007 and p=0.02 respectively), but not in *Bmal1* KO mice (red). N_{WT} = 5, $N_{Bmal1\ KO}$ = 6. Mean ± S.E.M. shown.

JTK_CYCLE analysis revealed significant diurnal rhythmicity of the relative abundances of Bacteroidetes and Firmicutes in WT mice (p=0.008 and p=0.02 respectively) but none in *Bmal1* KO mice (p=1 for both) (Figure 5.6 C, D). Accordingly, the ratio of Bacteroidetes to Firmicutes oscillated in WT mice, but not in *Bmal1* KO mice (Figure 5.6 B). The total bacterial load in both groups showed a similar fluctuating pattern, although those in *Bmal1* KO mice were depressed relative to WT mice (Figure 5.6 A). Thus, although the bacterial load was unaltered, *Bmal1* deletion abolished the diurnal oscillation of microbiota composition.

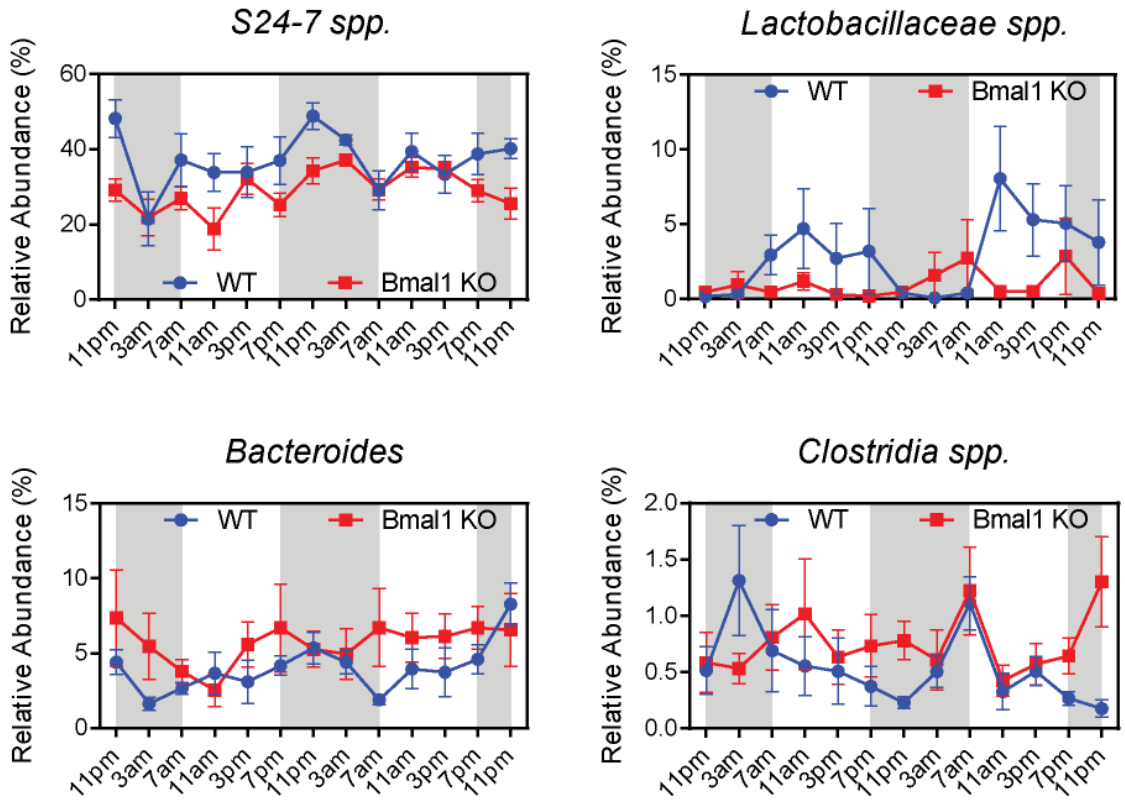

Figure 5.7 *Bmal1* deletion abolishes the diurnal oscillation at genus level in mice. WT mice exhibited diurnal oscillations during the light-dark cycle in the abundances of *S24-7 spp.* (p=0.04), *Lachnospiraceae spp.* (p=9.9E-05), *Oscillospira* (p=0.0005), *Bacteroides* (p=0.006), and *Clostridia spp.* (p=0.001). None of these genera oscillated in *Bmal1* KO mice. $N_{WT} = 5$, $N_{Bmal1\ KO} = 6$. Mean ± S.E.M. shown.

In WT mice, the relative abundances of *S24-7 spp.*, *Lactobacillaceae spp.*, *Baceroides*, and *Clostridia spp.* showed significant diurnal oscillation. However, in *Bmal1* KO mice, none of these oscillated (Figure 5.7).

5.3.3 *Bmal1* deletion alters the microbiota composition

The microbial community composition in *Bmal1* KO mice and WT mice was different based on presence-absence analysis (Figure 5.8 A) (unweighted UniFrac distance, p=0.03) but only showed a trend toward differences in the abundance-weighted analysis (weighted UniFrac distance, p=0.19). The degree of difference between genotypes did not depend on the time of day when the samples were collected (unweighted UniFrac, p=0.97). Comparison of abundances at the phylum level revealed a significant decrease in the relative abundance of Proteobacteria as well as a significant increase of phylum TM7 in *Bmal1* KO mice compared to those in WT mice (Figure 5.8 B). The decrease in abundance of Proteobacteria was driven by the reduction of two genera, *Helicobacter* and *Sutterella*, whereas the increase of TM7 abundance was driven by the expansion of *F16 spp.* (Figure 5.8 C, top and bottom). At the genus level, significant alterations in the relative abundances could be detected. *Bmal1* deletion was associated with significant increases of *Bacteroidales spp.*, *Rikenellaceae spp.*, and *Rikenella* abundances (Figure 5.8 C, top and middle), as well as significant decreases of *S24-7 spp.* abundance (Figure 5.8 C, bottom) within the phylum Bacteroidetes. It was associated with significant increases of *Clostridiales spp.*, *Clostridiaceae spp.*, and *Peptococcaceae spp.* abundances (Figure 5.8 C, upper and middle), and significant decrease of *Allobaculum* abundance (Figure 5.8 C, bottom) within the phylum Firmicutes.

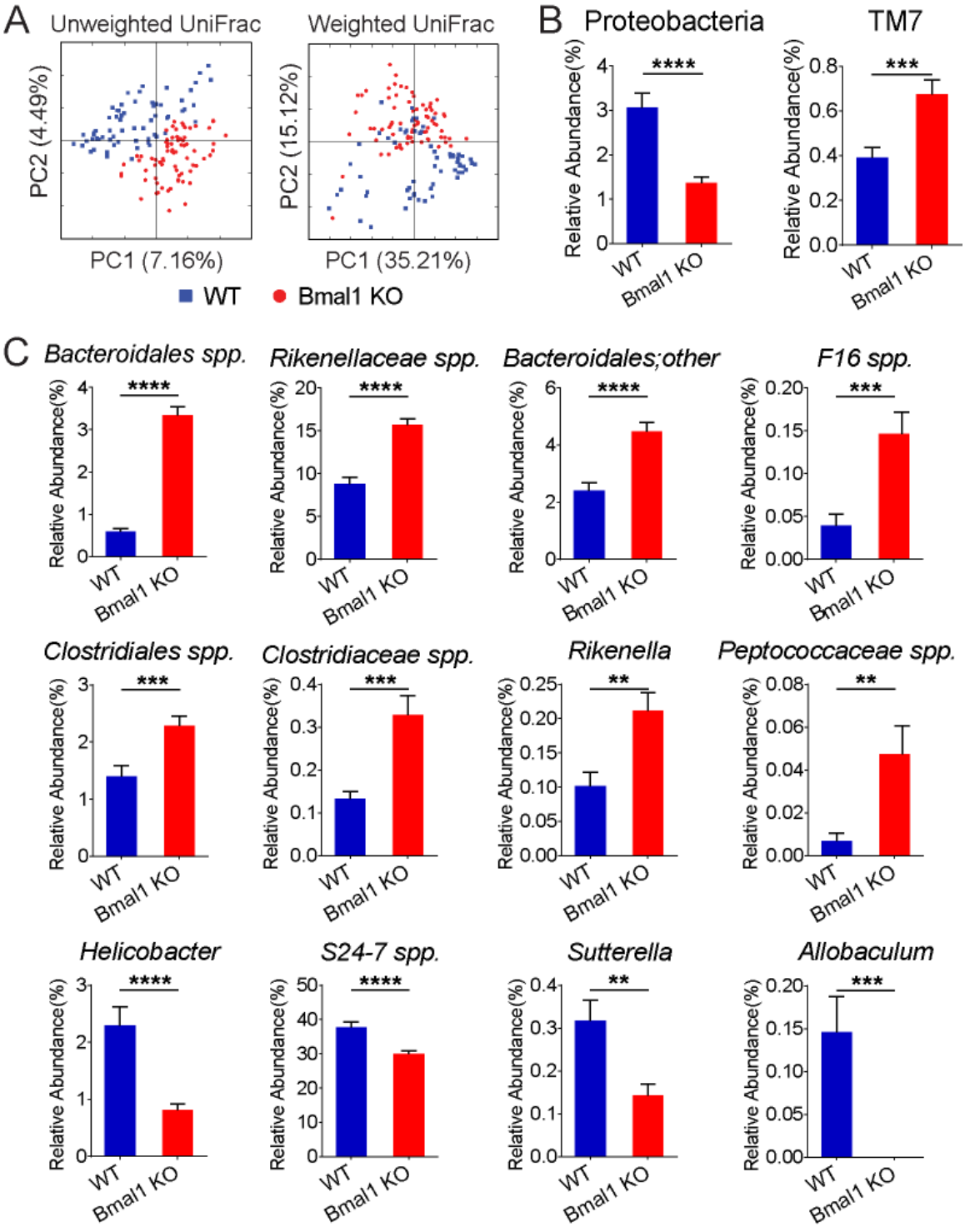
A
Unweighted UniFrac
Weighted UniFrac
PC2 (4.49%)
PC1 (7.16%)
PC2 (15.12%)
PC1 (35.21%)
WT
Bmal1 KO
B
Proteobacteria
TM7
Relative Abundance(%)
****
***
C
Bacteroidales spp.
Rikenellaceae spp.
Bacteroidales;other
F16 spp.
Clostridiales spp.
Clostridiaceae spp.
Rikenella
Peptococcaceae spp.
Helicobacter
S24-7 spp.
Sutterella
Allobaculum
**

Figure 5.8 *Bmal1* deletion alters bacterial relative abundances in fecal microbiota in mice. Fecal microbiota compositions from WT and Bmal1 KO mice were compared. **(A)** Fecal microbiota composition from individual WT mice (blue) and *Bmal1* KO mice (red) were clustered separately according to Principal Coordinate Analysis of Unweighted UniFrac distances (let) and Weighted UniFrac distances (right). The percentages of variation explained by principal coordinates PC1 and PC2 are indicated on the x and y axes, respectively. **(B)** The relative abundance of Proteobacteria was decreased in *Bmal1* KO mice. The relative abundance of TM7 and unassigned species (Other) was increased in *Bmal1* KO mice. **(C)** The relative abundances of bacterial genera were altered in *Bmal1* KO mice. $N_{WT} = 5$, $N_{Bmal1\ KO} = 6$. Mean ± S.E.M. shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Mann-Whitney test.

5.3.4 *Bmal1* deletion-induced changes in the microbiota are gender-dependent

Principal Coordinate Analyses also revealed gender differences in the microbiota composition (Figure 5.9 A) (p=0.026 for unweighted UniFrac; p=0.007 for weighted UniFrac). The effect of *Bmal1* deletion was the same for male and female mice. In male mice, *Bmal1* deletion was associated with a significant decrease of Proteobacteria (Figure 5.9 B) and increase of TM7 (Figure 5.9 C). In female *Bmal1* KO mice, however, these phyla were not changed, but Cyanobacteria (Figure 5.9 D) and Tenericutes (Figure 5.9 E) increased. *Bmal1* deletion-induced changes in female mice were not as substantial as those in male mice.

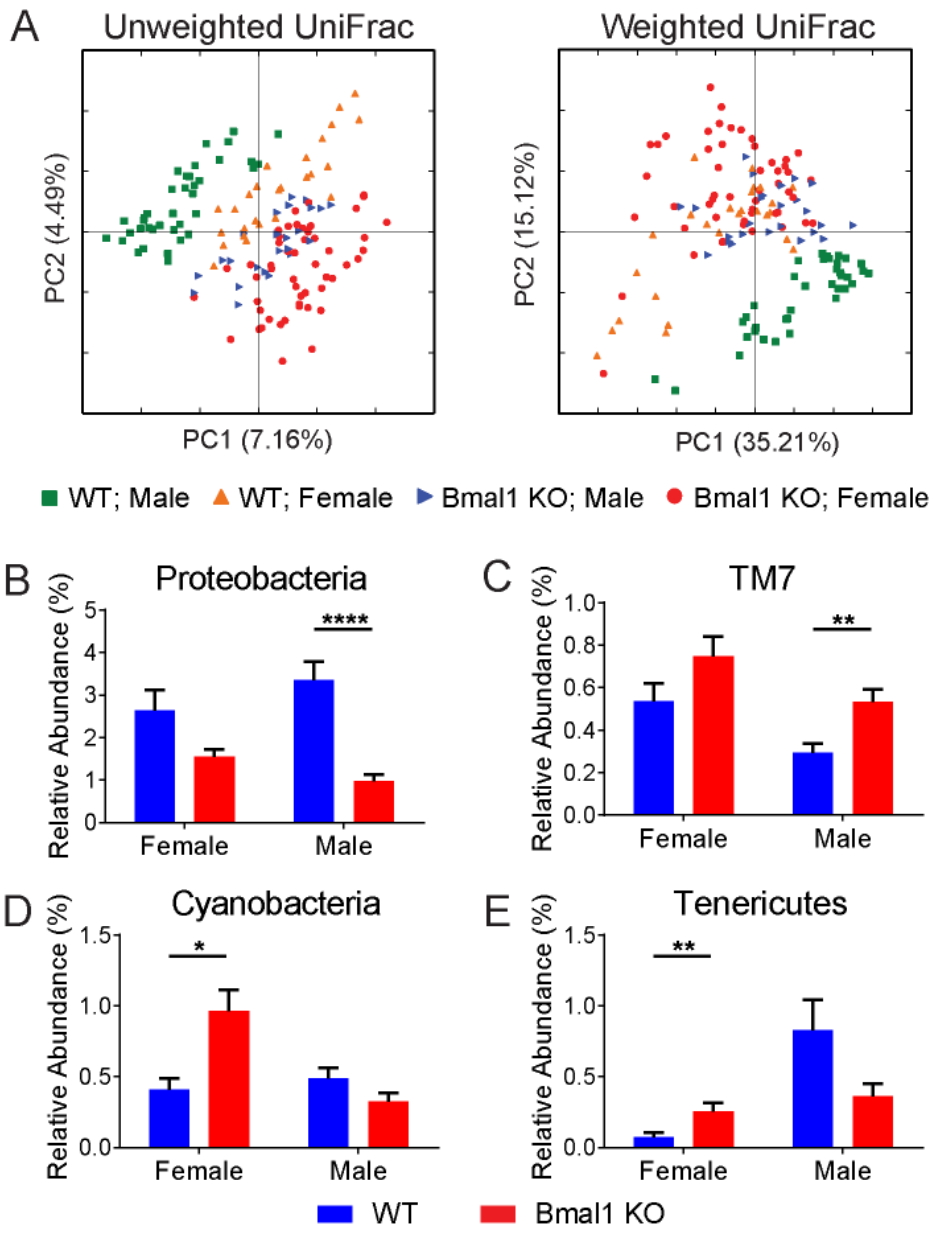
A
Unweighted UniFrac
Weighted UniFrac
PC2 (4.49%)
PC1 (7.16%)
PC2 (15.12%)
PC1 (35.21%)
WT; Male
WT; Female
Bmal1 KO; Male
Bmal1 KO; Female
B
Proteobacteria
Relative Abundance (%)
****
Female
Male
C
TM7
**
D
Cyanobacteria
*
E
Tenericutes
WT
Bmal1 KO

Figure 5.9 Intestinal microbiota compositions of male and female mice respond differently to *Bmal1* deletion. Fecal microbiota compositions from individual mice at all time points across the day were compared based on gender and genotype. **(A)** Fecal microbiota composition from individual male WT mice (green), female WT mice (orange), male *Bmal1* KO mice (blue), and female *Bmal1* KO mice (red) were clustered according to Principal Coordinates Analysis of Unweighted UniFrac distances (let) and Weighted UniFrac distances (right). Percentages of variation explained by principal coordinates PC1 and PC2 are indicated on the x and y axes. **(B -E)** Bacterial phyla abundances of WT (blue) and *Bmal1* KO mice (red) in female and male mice were compared. *Bmal1* deletion caused a decrease of Proteobacteria **(B)**, and an increase of TM7 **(C)** in male mice. Female mice showed increased Cyanobacteria **(D)** and Tenericutes **(E)** after *Bmal1* deletion. $N_{WT} = 5$, $N_{Bmal1\ KO} = 6$. Mean ± S.E.M. shown. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by Mann-Whitney test.

The microbiota composition of male *Bmal1* KO mice was more similar to female WT mice than to male WT mice (Mann-Whitney $p<0.001$ for unweighted and weighted UniFrac distance). In male *Bmal1* KO mice, the relative abundances of Proteobacteria, TM7, and Tenericutes were significantly altered compared to male WT mice, resulting in comparable levels to those in female WT mice (Figure 5.9 B, C, E).

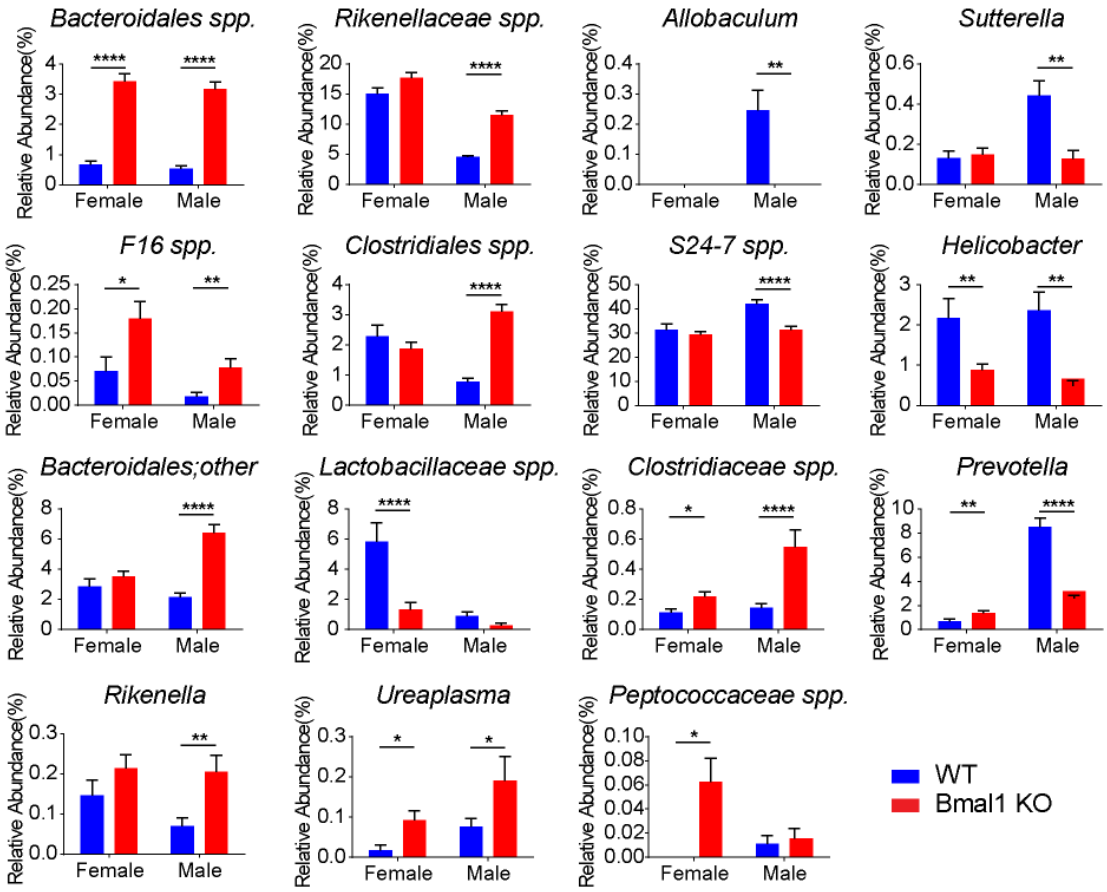

Figure 5.10 Differentially altered genera in female and male mice after *Bmal1* deletion. Bacterial genera abundances of WT (blue) and *Bmal1* KO mice (red) in female and male mice were compared. In female mice, *Bmal1* deletion was associated with increase of *Bacteroidales spp.*, *F16 spp.*, *Clostridiaceae spp.*, *Peptococcaceae spp.*, *Ureaplasma*, and decrease of *Helicobacter*, *Lactobacillaceae spp.*. In male mice, *Bmal1* deletion was associated with increase of *Bacteroidales spp.*, *Rikenellaceae spp.*, *Bacteroidales spp.*, *F16 spp.*, *Clostridiales spp.*, *Rikenella*, *Clostridiaceae spp.*, *Ureaplasma*, and decrease of *Allobaculum*, *S24-7 spp.*, *Helicobacter*, *Sutterella*, *Prevotella*. $N_{WT} = 5$, $N_{Bmal1\ KO} = 6$. Mean ± S.E.M. shown. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by Mann-Whitney test.

Examination at the genus level revealed consistent results (Figure 5.10). In male mice, *Bmal1* deletion caused significant increases of the relative abundances of *Bacteroidales spp.*, *Rikenellaceae spp.*, *F16 spp.*, *Clostridiales spp.*, *Rikenella*, and *Clostridiaceae spp.*, as well as significant decreases of the relative abundances of *Allobaculum*, *S24-7 spp.*, *Sutterella*, *Prevotella*, and *Lactobacillaceae spp.*. In female mice, *Bmal1* deletion resulted in significant increases of *Bacteroidales spp.*, *Clostridiaceae spp.*, *Peptococcaceae spp.*, *Prevotella*, and *Ureaplasma*, as well as significant decreases of *Helicobacter* and *Lactobacillaceae spp.*.

5.4 Discussion

The intestinal microbiota composition is subject to changes induced by ageing, use of antibiotics, diet, and disease (Zoetendal, de Vos 2014, Zhao 2013, Wu, Bushman & Lewis 2013, Agans et al. 2011, De La Cochetiere et al. 2005). Here we provide evidence that the microbiota exhibit daily fluctuations, and begin to specify the factors that influence these oscillations. We report that both the fecal microbial load and its composition oscillate diurnally in mice during the light-dark cycle, consistent with two reports on circadian variations of the fecal microbiota composition (Thaiss et al. 2014, Zarrinpar et al. 2014), but this study is the first to show variation in the fecal bacterial load as well. We also find, unexpectedly, that this oscillation is more pronounced in female mice than in male mice.

The relative abundance of both Bacteroidetes and Firmicutes oscillate diurnally (Figure 5.3). Bacteroidetes peaked several hours after the beginning of the dark phase and

Firmicutes peaked around the beginning of the light phase. Cyclical fluctuations were also detected in abundances of lower level taxa, including *Lachnospiraceae spp.*, *Oscillospira*, *Ruminococcaceae spp.*, *Clostridiales spp.*, *Clostridia spp.*, and *Anaeroplasma*. In the recent metagenomic study, several strains in the intestinal microbiota have been shown to have a functional β-glucuronidase and represent a major de-glucuronidation pathway, including several major species from Firmicutes and Bacteroidetes (Gloux et al. 2011) such as *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiaceae*. Interestingly, the relative abundances of these bacterial lineages undergo circadian variations according to our and other's studies. Together with our findings that the intestinal microbiota affects the pharmacokinetics and effect of indomethacin in mice partially via bacterial β-glucuronidase-mediated de-glucuronidation (Chapter 4), it is possible that the intestinal microbiota is partially contributing to the chronopharmacology of indomethacin in mice.

To probe the determinants of circadian variation, we assessed microbiota oscillation in mice lacking *Bmal1*, the central component of the forward limb in host circadian clock. We found that the conventional *Bmal1* deletion dampens the daily oscillation in both genders. We also report that conventional *Bmal1* deletion shifts the microbiota configuration, and that the two genders are affected differently.

Thaiss and colleagues found that the rhythmicity of microbiota composition is mostly lost after ablation of *Per1* and *Per2* genes, which are key components of the host molecular clock (Thaiss et al. 2014). A complication in the interpretation is that deletion of *Per1* and *Per2* has pleiotropic effects extending beyond just circadian behavior, such as

involvement in cell growth and cell cycle regulation (Yu, Weaver 2011). Here we report that *Bmal1* deletion, which leads to arhythmicity without pleiotropic effects, also abolishes the diurnal oscillation in fecal microbiota. Therefore, both activator (*Bmal1*) and repressor (*Per1,2*) genes in the core clock of the host are fundamental to the oscillation of fecal microbiota composition. This is strengthened by the finding that knockout of *Bmal1* and the *Per1,2* show similar phenotypes with microbiota rhythmicity. The mechanism underlying the effect needs further investigation.

Therefore, it is essential to maintain a functional host circadian system, in order to maintain a healthy microbiota composition. Factors affecting the host circadian system, such as shift-work or chronic jet-lag, would have an influence on the microbiota circadian variation and composition, hence affecting the pharmacokinetics and efficacy of indomethacin.

Rhythmicity of intestinal microbiota composition may also be affected by feeding behaviors. Mice are active in the dark phase (when lights are off from 7pm to 7am in our housing facility) and they consume the majority of their food during the first several hours of the dark phase (Paschos et al. 2012). Fasting is associated with expansion of Bacteroidetes and ingestion of food with an increase of Firmicutes (Costello et al. 2010, Crawford et al. 2009). During the day, when the mice are resting and consuming less food, Bacteroidetes are predominant, reaching the highest abundance towards the end of the light phase. When the lights go off, mice start eating. This is associated with the expansion of Firmicutes and proportional reduction of Bacteroidetes. By restricting the feeding of *Per1/2* KO mice to dark phase or light phase, the diurnal microbial oscillation

was restored (Thaiss et al. 2014). Partial restoration of microbial oscillation is evident by consolidating high-fat diet feeding to dark phase (Zarrinpar et al. 2014). However, since circadian rhythms of host behavior and physiology can be entrained by daily cycles of restricted feeding (Stephan 1984, Boulos, Rosenwasser & Terman 1980), it is possible that consolidation of feeding to the dark phase only partially restored the circadian rhythm of the host. Hence, it remains to be determined whether the restoration of microbial oscillation is due to a direct influence of feeding, due to restoration of the host circadian clock, or due to the combination of the two.

The influence of feeding on microbiota structure may result from varying nutrient availability. Glycans and polysaccharides from the diet and from the host mucus are essential carbon sources for growth of intestinal bacteria (Koropatkin, Cameron & Martens 2012). Unlike the host-derived glycans which are constantly available, dietary glycans fluctuate in both composition and abundance due to feeding. As an adaption to this, several bacterial strains in the Bacteroidetes phylum evolved to utilize host mucosal glycans when dietary glycans are in short supply (Martens, Chiang & Gordon 2008, Sonnenburg et al. 2005). Thus, bacterial strains that use only dietary glycans become proportionally reduced when the host is fasting, whereas bacteria that can use host-derived glycans, such as Bacteroidetes, increase. This may explain the expansion of Bacteroidetes during fasting and the increase of Firmicutes after food intake.

We found that *Bmal1* both mediates circadian rhythms and influences microbiota composition. *Bmal1* deletion shifted the microbiota configuration toward a phenotype that may be pro-inflammatory. For example, an increase of TM7 has been shown to be

associated with the development of periodontal disease (Brinig et al. 2003) as well as the inflammatory gastrointestinal disorders (Kuehbacher et al. 2008), probably by changing the growth environment for competing bacteria. Subphylum changes also indicate the likelihood of mild inflammation. An increase in *Rikenellaceae* abundance has been found as a result of high-fat diet feeding (Kim et al. 2012) and during the pathological progression of inflammatory bowel disease (Alkadhi et al. 2014). An increase of *Clostridiaceae* had been found in patients following ileal pouch-anal anastomosis (Scarpa et al. 2011, Tannock et al. 2012), in patients with ulcerative colitis and Crohn's disease (Zhang et al. 2013), and also in mice during the progression of lupus (Zhang et al. 2014b). A decrease of *Allobaculum* has also been found in mice fed a high-fat diet (Qiao et al. 2014). However, Proteobacteria, associated with host inflammation (Mukhopadhya et al. 2012), were decreased after *Bmal1* ablation. Our findings do not presently identify the mechanism through which *Bmal1* affects the configuration of the microbiota and it is also unknown whether deletion of the period genes influenced taxonomic composition (Thaiss et al. 2014).

Our study highlights the effect of gender on the composition of the fecal microbiota. Although microbiota in both males and females exhibited diurnal oscillations, females showed more significant rhythmicity than males (Figure 5.3). Oscillation in both genders depended on a functional host circadian clock, since *Bmal1* deletion abolished the rhythmicity irrespective of gender. Thaiss and colleagues also reported near-complete loss of diurnal oscillation of microbiota in *Per1/2* deficient mice, irrespective of gender

(Thaiss et al. 2014). Therefore, although secondary to the effect of the circadian clock, host gender also shapes the rhythmicity of composition.

We also found that gender conditioned the impact of *Bmal1* deletion on microbiota composition (Figure 5.9, Figure 5.10), indicating an interaction between gender and genotype. There are some previous suggestions of the relevance of gender to the microbiota. For example, male and female microbiota diverge after puberty in mice and this reflects the gender bias in expression of autoimmune diseases, such as type 1 diabetes (Markle et al. 2013, Yurkovetskiy et al. 2013). Sexual dimorphism in the microbiota has also been reported in macaques (McKenna et al. 2008). Although the mechanism remains unclear, a bidirectional interaction between microbiota and host hormone levels seems likely, since the divergence between male and female microbiota can be reversed by male castration (Markle et al. 2013). Moreover, differential effects of dietary manipulation are conditioned by gender in fish, mice and potentially humans (Bolnick et al. 2014).

In summary, the composition of the fecal microbiota exhibits diurnal oscillation that requires a functional host circadian clock, as indicated by comparison of the *Bmal1* deletion. *Bmal1* also had a role in specifying the composition of the microbiota, and this was affected by host gender. Our findings thus specify pathways of host regulation of the composition of fecal microbiota and motivate exploration of the host circadian clock and microbial circadian rhythmicity. Our study and previous work (Thaiss et al. 2014, Zarrinpar et al. 2014) suggest the need to include consideration of animal gender and genotype, feeding pattern, and timing of sample collection in studies of the microbiota.

Moreover, our study provides initial evidence documenting the involvement of intestinal microbiota in the chronopharmacology of indomethacin.

Chapter 6 Conclusions and Future Directions

6.1 Conclusions

This thesis supports the following conclusions:

1. In untreated mice, the microbial load and composition vary considerably from the proximal to the distal end of the intestine, as well as between the luminal content and mucosal tissue. The microbiota composition in the small intestine has greater inter-individual variation in both luminal content and mucosal tissue.
2. A single oral dose of indomethacin, which has the potential to induce small intestinal damage, altered microbial diversity in the distal intestine and caused compositional changes along the intestine without affecting the microbial biomass.
3. Indomethacin induces changes in microbial diversity and composition in feces, shifting the microbial community towards a pro-inflammatory phenotype.
4. The half-life and volume of distribution of indomethacin was significantly reduced in mice treated with antibiotics. This is partially due to impaired de-glucuronidation mediated by bacterial β-glucuronidase.
5. Both the fecal microbial load and its composition oscillate diurnally in mice during the light-dark cycle. This oscillation is regulated by host circadian clock at an early stage of life.

6.2 Future directions

The research in this thesis prompts new questions and opens the door for future investigations.

We established the association of indomethacin administration with altered intestinal microbiota composition. The expansion of *Peptococcaceae* and *Erysipelotrichaceae* has been previously been associated with inflammatory disease (Harris et al. 2014, Zhang et al. 2009, Zhu et al. 2014, Kaakoush et al. 2015). Similarly, the increased ratio of Firmicutes to Bacteroidetes in the fecal microbial community has been associated with obesity (Ley et al. 2005, Turnbaugh et al. 2006). However, whether the alterations in intestinal microbiota composition play a role in the pathogenesis of indomethacin-induced intestinal damage and whether this effect is through direct interaction or indirect interference needs further study. Since COX-2 specific inhibitors showed a better gastrointestinal safety profile than tNSAID comparators (Bombardier et al. 2000, Silverstein et al. 2000), this question can be partially addressed by comparing the influences of NSAIDs that favor COX-1 inhibition, such as naproxen or other compounds, versus that of NSAIDs that are specific for COX-2 inhibition, such as celecoxib (FitzGerald, Patrono 2001).

In addition, the mechanism via which indomethacin causes these changes are not revealed in this study. Further experiments are needed to assess the following: i) whether or not indomethacin is directly affecting the growth of intestinal bacteria by providing a nutritional source that can be uniquely utilized by specific species; ii) whether or not

COX-1 and COX-2 inhibition is involved in indomethacin-induced compositional changes in intestinal microbiota, and if so, what are the downstream mediators; iii) whether COX-1 or COX-2 inhibition is the major driver of indomethacin-induced changes. The first question can be addressed by in vitro screening of bacteria whose growth can be affected by the presence of indomethacin in the culture media. The second question can be addressed by investigating the intestinal microbiota composition in genetically modified mouse models, such as those lacking COX-1 or COX-2. Further, tissue-specific knockout mice, can be used to elucidate the influence of COX-1 and COX-2 enzymes. The third question can also be addressed by using pharmacological probes.

This thesis research revealed the diurnal oscillation of several bacterial strains that has been predicted to have functional β-glucuronidases activity. Given the chronopharmacology of indomethacin, it is important to study whether the circadian effect is attributable to the diurnal oscillation of intestinal microbiota. This can be addressed by: i) studying the pharmacokinetics of indomethacin in germ-free mice or antibiotic-treated mice receiving indomethacin in the mornings versus in the evenings; ii) studying the pharmacokinetics of indomethacin in gnotobiotic mice co-colonized with bacterial strains with predicted β-glucuronidases activity.

This thesis research investigated the acute effect of indomethacin administration. However, long-term use of NSAIDs by patients is quite common. Hence, it is worthwhile to investigate the influence of indomethacin on intestinal microbiota after chronic

administration. Further, naproxen and celecoxib can also be studied to differentiate COX-1 inhibition versus COX-2 inhibition.

Bacteria-mediated de-glucuronidation has been identified as one potential mechanism through which intestinal microbiota affect the pharmacokinetics and efficacy of indomethacin. To further confirm the involvement of intestinal bacteria, the following approach can be used: i) compare the pharmacokinetics of indomethacin in germ-free mice versus conventional mice; ii) compare the pharmacokinetics of indomethacin in mice treated with or without bacteria-specific inhibitors for β-glucuronidases developed previously (Roberts et al. 2013, Saitta et al. 2014).

Co-administration of antibiotics with NSAIDs is common in orthopedic patients. Therefore, it is important to study whether the pharmacokinetic and pharmacodynamics changes reported in this thesis research would be generalized to human populations and whether this influence would limit the efficacy of NSAIDs in these patients.

The importance of intestinal microbiota is more and more appreciated in the past several years. This thesis research is the first to document the bidirectional interactions between host-targeting medication and intestinal microbiota, phenomena that are closely relevant to human health and disease.

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